Assessing genetic variability and physiological traits conferring drought stress tolerance in cereals

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List of Abbreviations

ABA	abscisic acid
ATP	adenosine triphosphate
BSM	basic salt media
DDI	drought damage index
DH	double haploid
DW	dry weight
FAO	Food and Agriculture organization of the United States
Fv/Fm	maximal quantum efficiency of PSII
FW	fresh weight
Gs	stomatal conductance
LWP	leaf water potential
MIFE	microelectrode ion flux estimation
PCA	principle component analysis
PSII	photosystem II
ROS	reactive oxygen species
RWC	relative water content
SD	stomatal density
SE	standard error

Abstract

Drought is the most devastating environmental stress affecting plant growth and productivity. Almost half of the terrestrial land surface in the world i.e., 6.45 billion hectares, is composed of dry land. Global warming is expected to further exacerbates the intensity of drought and could result in significant (over 75%) losses in agricultural production worldwide. Wheat and barley are amongst most important cereal crops in the world. Wheat is moderately drought tolerant while barley is classified as relatively drought tolerant. Given the extent of dry land in the world and predicted population growth to 9.3 billion by 2050, creating drought tolerant barley and wheat germplasm is the ultimate priority of the plant breeders. However, the progress in breeding for drought tolerance is significantly handicapped by the lack of convenient and reliable phenotyping methods to screen a large germplasm.

Drought tolerance is a complex trait. As a result of a large genetic diversity of barley and wheat, plants show a plethora of morphological, physiological and biochemical responses to drought stress and rely on different adaptive mechanisms. These adaptive mechanisms include mainly stomatal regulations, signalling pathways, root related traits and osmoregulation. Different plant species cope with drought stress either by closing their stomata to prevent water loss or maintaining relative water content by osmotic adjustment by increased accumulation of organic and inorganic osmolytes. Taken together, it remains still elusive which trait should be targeted for drought tolerance in barley and wheat. Addressing the above issues, thirty varieties of barley (*Hordeum vulgare* L.), 18 bread wheat (*Triticum aestivum* L.) and two durum wheat (*Triticum durum*) varieties were collected from different geographical locations for the present study. Two different type of glasshouse experiments were performed. The first experiment was conducted in large tanks applying drought treatment to plants at three to four leaf stage by withholding irrigation for seven weeks. The plants were evaluated based on the visual damage at 3rd, 5th and 7th week of drought imposed and a visual score (0-10, 0= no visual symptoms to stress and 10= all plants are dead) was given to the plants by counting the number of chlorotic and necrotic leaves. The second experiment was performed in the pots and at two to three leaf stage, when seedlings were subjected to three irrigation regimes: control (100% field capacity) and two stress treatments (25% and 12% of full field capacity). After six weeks of drought imposed, plant agronomical (shoot fresh weight, shoot dry weight) and physiological

(chlorophyll content, chlorophyll fluorescence, stomatal conductance and relative water content) characteristics were measured.

We also assessed the suitability of different screening techniques to determine drought tolerance. Visual evaluation based on drought damage index provided a simple and feasible approach to measure the tolerance to water stress as it does not require any special equipmental expertise and can be used for screening on a large scale. SPAD and *Fv/Fm* measurements were quick and non-invasive and deemed as suitable indices for measuring drought tolerance. However, maintaining field capacity on daily basis in the pot experiment was labour-intensive and time-consuming job and not recommended for a large-scale screening.

Based on drought damage index (DDI), barley genotypes were divided into four groups i.e., tolerant, moderately tolerant, moderately sensitive and sensitive. In the second experiment, biomass and physiological traits were evaluated. Stomatal conductance, chlorophyll content (SPAD), chlorophyll fluorescence (Fv/Fm), relative water content (RWC) were significantly reduced for the plants grown under 25% and 12% of full field capacity. The genotypes showed similar trend for drought tolerance and susceptibility in both the experiments. Cultivars Numar, Flagship, ZUG293 and X026 were referred as highly drought tolerant (DDI <6.5 and accumulated high biomass) and Franklin and Gairdner (DDI >9 and accumulated low biomass) as highly drought sensitive. A significant correlation was found between shoot dry weight of plants grown under 12% field capacity and chlorophyll content (SPAD), chlorophyll fluorescence (Fv/Fm), stomatal conductance (Gs), relative water content (RWC) and fresh weight (FW) of plants grown under 25% and 12% field capacity irrigation regime.

In wheat, significant genotypic differences were observed among all genotypes under drought stress. Based on both screening experiments, genotypes Albidum24, Tainong292 and Mahon Demias were classified as drought tolerant with DDI<6.5, and relatively high biomass, SPAD and Fv/Fm values. Genotypes Onohoiskaja4, Kord Cl Plus (DD1>7.5) and Zhemgmai9023 (DDI>9) had lowest biomass and relative water content under severe stress (12% of full field capacity) and were deemed as drought sensitive. Another finding of note was that barley showed rather different strategies dealing with drought as compared with wheat. In wheat, the tolerance was achieved by closing stomata (low relative Gs values- 19% to 0.6%) while in barley, the plants

were able to open their stomata (high relative Gs values- 23% to 0.7%) for a longer time and maintained higher water content due to more efficient osmotic adjustment.

Based on a large screening of barley genotypes for their drought tolerance, seven contrasting genotypes (Numar, ZUG293-tolerant, Commander, Fleet, X123-moderately tolerant, Franklin, Gairdner- sensitive) were selected for the third experiment. The experiment was conducted under glasshouse conditions, with genotypes grown under 100%, 25% and 12% field capacity to study various physiological traits and linking the overall drought tolerance with changes in plant water related traits, stomatal characteristics and the contribution of organic and inorganic osmolytes towards the root and shoot osmotic adjustment. The overall drought tolerance was positively correlated with root length, stomatal conductance, relative water content, stomatal density, root K^+, root Cl^-, leaf Cl^-, total soluble sugars, total amino acids of plants grown under 12% field capacity irrigation regime. However, leaf water potential, leaf K^+ and Na^+ content of plants grown under 12% field capacity irrigation showed no correlation with drought tolerance. Taken together, these results suggest drought tolerant genotypes had higher root K^+ and Cl^- and organic osmolytes content as well as high stomatal conductance (Gs), stomatal density (SD), relative water content (RWC) and root length (RL) under drought stress. The relative contribution of inorganic (K^+, Na^+, Cl^-) and organic osmoles (total soluble sugars-TSS, total amino acids-TAA) towards the leaf and osmolality of plants under drought stress was in the order Cl > K > TSS > TAA > Na. The relative contribution by Cl^- was also the highest towards the root osmolality, followed by K^+ and Na^+.

Abscisic acid (ABA) regulates various molecular events in response to water deficit in plants. To elucidate the abscisic acid mediated signalling in barley roots and ionic mechanisms of osmoregulation, the non-invasive ion-selective microelectrode measurements (the MIFE technique) was used. Transient ion fluxes in response to hyperosmotic stress were compared for seven barley genotypes contrasting in drought tolerance (Numar, ZUG293-tolerant, Commander, Fleet, X123-moderately tolerant, Franklin, Gairdner- sensitive). All the genotypes had uptake of K^+ and Cl^- under hyperosmotic stress (200mM mannitol) in the root mature zone and net uptake of K^+ and Cl^- was positively correlated this drought tolerance of the cultivar. However, there was an efflux of Na in response to hyperosmotic stress. Long term (48 hours) of hyperosmotic stress caused further increase in the uptake of K^+ and Cl^- in drought tolerant genotypes. Another set of experiments was performed to measure membrane

potential in the root mature zone. Drought tolerant genotypes were able to maintain more negative membrane potential values as compared to moderately tolerant and sensitive genotypes. Abscisic acid application increased Cl^- uptake in the roots of ZUG293 and Franklin whereas there was no significant effect of ABA on K^+ and Na^+ in both the genotypes suggesting the role of ABA in regulating ions in roots is opposite to the role of ABA in guard cells.

Overall, this work has screened and identified barley and wheat genotypes contrasting in their drought tolerance. These genotypes are recommended for mapping double haploid (DH) population to reveal the QTLs responsible for drought tolerance in these species. We also found that barley has rather different drought tolerance mechanisms compared to wheat. This study also recommended some rapid and convenient screening methods to screen a large germplasm for drought tolerance. Inorganic osmolytes mainly Cl and K made the highest contribution to root and leaf osmolality. Leaf K^+ and Na^+ did not correlate with drought tolerance, however, root K^+ and Cl^- correlated with drought tolerance. Therefore, we could recommend breeders to select genotypes which are drought tolerant based on root inorganic ions such as K^+ and Cl^-. Hyperosmotic stress induced by mannitol caused a significant uptake of K^+ and Cl^- in drought tolerant genotypes compared to moderately tolerant and sensitive suggesting the activation of HvAKT1 uptake channels and HvHAK1 (HAK/KUP/KT transporters) in tolerant genotypes, as evident from the fact that they were able to maintain more negative membrane potential under hyperosmotic stress. Both HvAKT1/HvHAK1 activated by membrane hyperpolarization brought about by increased uptake of Cl^-. Another important finding is that effects of abscisic acid (ABA) on K^+ transport in root cells was different from that in guard cells. Application of ABA induced an increase in Cl^- in both tolerant and sensitive genotypes and caused no significant effect on cations (K^+ and Na^+). This ABA mediated Cl^- uptake and hyperpolarization of PM both are intrinsically linked with H^+-ATPase activity. In the future, more comprehensive studies on the function of plasma membrane H^+-ATPase in response to hyperosmotic stress and ABA needs to be done.

Chapter 1. Literature Review

1.1 Drought as an issue and economic penalties

Drought is a major problem associated with the global warming. Almost half (47%) of the terrestrial land surface in the world i.e., 6.45 billion hectares, is composed of dry land. One billion hectares are hyper-arid and 5.45 billion hectares are made up of arid, semi-arid and sub-humid areas (Fig 1.1). By the year 2025, 65% of world population will be living in drought-affected environment, and about 1.8 billion people will face a complete water scarcity (Arash Nezhadahmadi, 2013).

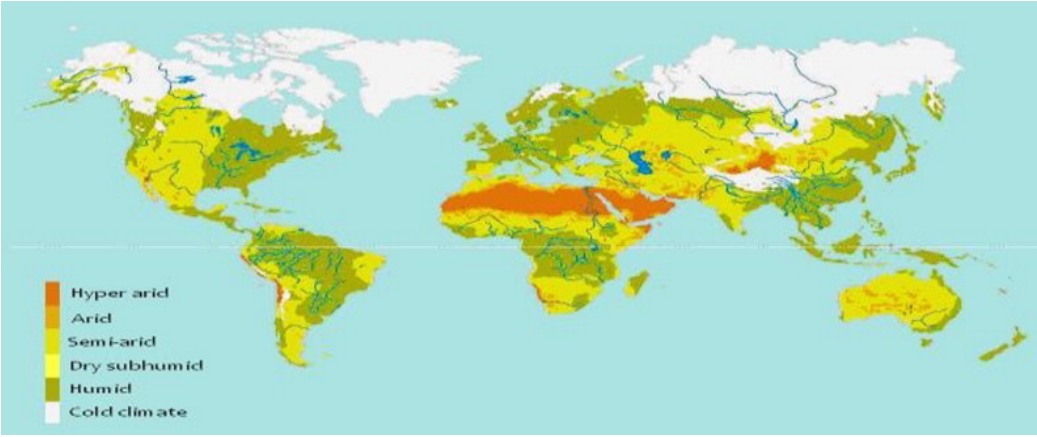

Figure 1.1 Geographical distribution of drought affected areas in the world (Karim and Rahman, 2015)

The effects of drought stress are expected to increase further with current climate change trends and a growing water crisis (Hasanuzzaman et al., 2013). The climatic models predict that global warming will further escalate drought as a result of increasing evapotranspiration (Cook et al., 2007) though there are likely to be large regional differences (Metz et al., 2007), with both frequency and intensity increasing from 1 to 30% in an extreme drought land area by 2100 (Fischlin, 2007). Plants undergo drought stress either due to the curtailment of water to the roots or when the transpiration exceeds to the threshold limit. Plant dehydration symptoms are curling or rolling of leaves, followed by yellowing and browning of leaves. The main consequences of drought in crop plants are reduced rate of cell division and expansion, leaf size, stem elongation and root proliferation, and disturbed stomatal oscillations,

plant water and nutrient relations with diminished crop productivity, and water use efficiency (Farooq et al., 2009a).

For drought, factors like severity, timing and duration of stress are pivotal. Drought-induced yield reduction has been reported in many crop species, which depends upon the severity and duration of the stress period. At vegetative growth stage, drought definitely causes economic crop losses but during reproductive and grain filling stage it is more devastating (Tab 1.1).

Table 1.1 Economic yield reduction by water stress in some major field crops

Crop	Growth level	%age yield decline	References
Barley	Seed filling	49-57	Samarah, 2005
Canola	Reproductive	30	Masoud Sinaki et al., 2007
Chickpea	Reproductive	45-69	Nayyar et al., 2006
	Late ripening	49-54	Samarah et al., 2009
	Anthesis	27-40	Mafakheri et al., 2010
Common	Reproductive	58-87	Martinez et al., 2007
beans	Flowering	49	Rosales Serna et al.,2004
	Pod filling	40	Ghanbari et al., 2013
Cowpea	Reproductive	60-11	Ogbonnaya et al., 2003
Faba bean	Grain filling	68	Ghassemi-Golezani and Hosseinzadeh-Mahootchy, 2009
Lentil	Reproductive	24	Allahmoradi et al., 2013
	Pod development	70	Shrestha et al., 2006
Maize	Grain filling	79-81	Monneveux et al., 2006
Maize	Reproductive	63-87	Kamara et al., 2003
Maize	Reproductive	70-47	Chapman and Edmeades, 1999
Maize	Vegetative	25-60	Atteya, 2003
Maize	Reproductive	32-92	Atteya, 2003
Mashbean	Flowering	31-57	Baroowa and Gogoi, 2014
	Reproductive	25	Baroowa and Gogoi, 2013
Pigeonpea	Reproductive	40-55	Nam et al., 2001
Rice	Reproductive	24-84	Venuprasad., 2007
Rice	Reproductive (mild stress)	53-92	Lafitte et al., 2007
Rice	Reproductive (severe stress)	48-94	Lafitte et al., 2007
Rice	Grain filling (mild stress)	30-55	Basnayake et al., 2006
Rice	Grain filling(severe stress)	60	Basnayake et al., 2006
Soybean	Grain filling	42	Maleki et al., 2013
	Reproductive	46-71	Samarah et al., 2006

1.2 Physiological constraints imposed by drought

Drought stress induces myriad of changes in physiological processes. The most critical one include stomatal closure, arrest of photosynthesis, decrease in nutrients uptake and oxidative damage. All these changes results in a reduced plant growth and productivity.

1.2.1 Stomatal closure and limitation of photosynthesis

Stomata are specific epidermal structures comprising of two guard cells around a pore. Each stoma is a molecular valve that acts in gas exchange, mostly CO_2 and O_2, which is vital for optimal photosynthesis and which limits water loss by regulating the transpiration level. Stomatal closure occurs through the turgor fluctuations of guard cells surrounding the stomata as when the water level in the cells lessened to the point where osmotic pressure is dropped, the guard cells are deprived of the turgor pressure and shrinks causing the stomata to be closed (Brodribb and McAdam, 2011; Casson and Hetherington, 2010; Clauw et al., 2015a). Under severe drought stress the initial reaction of most of the plants is the closure of their stomata. This rapid response is modulated by a complicated network of signaling pathways, in which the principal and the best-known role is played by abscisic acid (ABA) alongside with jasmonates (JA), ethylene, auxins, and cytokinins. Generally, ABA and JA are positive controllers of the stomatal closing, while auxin and cytokinins are positive regulators of stomatal opening. The activity pattern of ethylene is not clear as it can perform as a positive or negative regulator, depending upon the tissue and conditions (Nemhauser et al., 2006).

At the time of stomatal closure, reduced gas exchange results in the depletion of the photosynthesis, while decreased transpiration lessen down the water loss from leaves. For the growth of plants, it is pivotal to get water from the soil and CO_2 from the air to be used in the photosynthesis. This is done through the stomatal openings that take up CO_2 with the simultaneous transpiration. Water transpiration from the leaves forces the water uptake by the roots and move through xylem. Transpiration and CO_2 uptake is only possible when the stomata are open. When the stomata are closed under drought, CO_2 uptake become reduced, and transpiration declines (Arve et al., 2011). The CO_2 limited availability due to stomatal closure may also induce impairment to photosystem II. In addition, the imbalance between reactive oxygen species and antioxidant enzymes influences the photosynthetic potential of plants through higher

oxidation of proteins, membrane lipids, and other cellular characteristics Fig 1.2 (Farooq et al., 2009a).

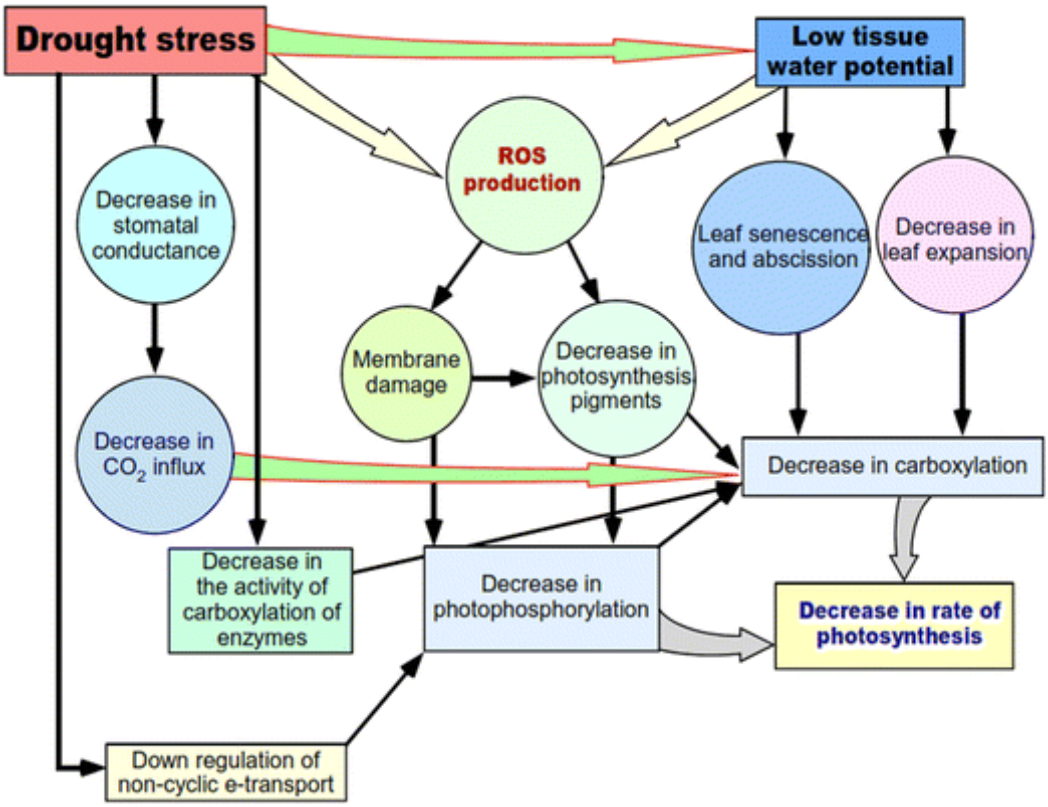

Figure 1.2 Effect of drought stress on photosynthesis. Drought stress reduce the tissue water status, which suppresses the leaf development and accelerates the leaf senescence and abscission resulting in decrease in photo-assimilatory size, and thus, carboxylation is decreased. Drought disturbs the balance between the production of reactive oxygen species (ROS) and the antioxidant defense causing accumulation of ROS, which induces oxidative stress. Drought also induces stomata closure, which limits the CO_2 influx. Decrease in CO_2 not only reduces the carboxylation directly but also directs more electrons to form ROS. Under severe drought, activities of carboxylation enzymes are reduced. Under drought stress, non-cyclic electron transport is downregulated to match the reduced requirements of NADPH production and thus reduces the rate of photophosphorylation (Farooq et al., 2009a)

1.2.2 Reduction in nutrient uptake

Uptake of most mineral nutrients are dependent on soil moisture to move through the soil matrix and be taken up by plants. The potential capability of plant roots to absorb water and nutrients generally decrease in water stressed plants.

Nitrogen

Nitrogen is an essential nutrient for optimal plant development as it acts as a key catalyst to support photosynthesis allowing plants to use sunlight and converting it into glucose which consequently gives plant energy. It is also a major constituent of nucleic acids, proteins and organic compounds required for healthy plant growth. Nitrogen is available in the form of NO_3^- (nitrate) or NH_4^+ (ammonium) ions to the plants. Most ammonium transforms into organic compounds in the roots. On the other hand, nitrate promptly moves into the xylem and can be stored in the vacuoles of roots, shoots and other storage organs (Barker and Pilbeam, 2015). The conversion from nitrate to ammonium is mediated by the enzymatic processes in which NO_3^- is reduced to NO_2^- catalyzed by enzyme nitrate reductase present in the non-organelle portions of the cytoplasm using energy and reductant of photosynthesis (Maynard, 2007). Drought reduces nitrate reductase activity in different species such as tomato, cowpea, cumin and barley (Krcek et al., 2008; Sepehr et al., 2012; Silveira et al., 2001; Sivakumar et al., 2014). Drought also reduces biological availability of ammonium and nitrate, as their delivery to the root surface is driven mainly by the bulk water flow and, therefore, is strongly influenced by the plant transpiration. Nitrogen deficiency increased a strong sensitivity of stomata to drought. When the soil faces a prolonged period of drought, nitrogen deficiency occurs which rapidly inhibits plant growth and leads to chlorosis. Almost 50% of all nitrogen in the leaf is directly involved in the process of photosynthesis, thus if N supply is insufficient due to drought stress, photosynthesis is decreased by reducing the leaf area and photosynthesis rate as well as accelerating leaf senescence (Bänziger, 2000; Kamara et al., 2014; Wang et al., 2016).

Phosphorus

Phosphorous is an essential plant nutrient as a component of nucleic acid structure that modulate protein synthesis in plants. Phosphorous plays a central role in cell division and the growth of new tissues. Phosphorus exists in organic forms such as non-decomposed plant and animal residuals and organic matter in soil as well as inorganic form usually linked with aluminium (Al), iron (Fe) and calcium (Ca). Soil moisture limitation reduced soil P diffusion and transport in plants (Suriyagoda et al., 2014). Phosphorous delivery to the root surface relies on its diffusion in the soil matrix, and thus is strongly reduced under drought conditions, as a result of decreased mobility (Fawecett and Smith, 2009; Faye et al., 2006). Maximizing the ability of the root to

absorb P from the soil is one of the main mechanisms to cope with the P deficiency in water limited conditions (He et al., 2017).

Higher P acquisition by plants relies on root morphology and architecture such as: (1) substantial root branching, (2) greater root length density and a maximum fraction of roots in surface soil layers, (3) higher production of thin roots, and (4) partitioning of more plant biomass to the root system (Lynch, 2011).Thus, altering the morphology and architecture of roots is a powerful way for crop plants to maximize root absorption for P acquisition.

Potassium

Potassium (K) is a vital nutrient that affects most of the biochemical and physiological processes such as protein synthesis, osmoregulation, stomatal movement, photosynthesis and enzyme activation that influence plant growth and metabolism. During drought stress, root growth and the rates of K^+ diffusion in the soil towards the roots are both restricted, thus limiting potassium acquisition (Mengal et al., 2006; Wang et al., 2013). The resulting lower K concentrations can further reduce the plant resistance to drought stress. Maintaining adequate potassium supply is, therefore, critical for plant drought resistance that can enhance the total dry mass accumulation of crop plants under drought stress in comparison to lower K concentrations. This finding might be attributable to the stomata regulation by K and corresponding higher rates of photosynthesis. Moreover, K is also essential for the translocation of photoassimilates from source to sink (Römheld and Kirkby, 2010). Water conditions in plants influence the potassium accumulation in leaves and interact with K nutritional status in some plants (Mengal et al., 2006). The adequate potassium supply is also essential in maintaining cell membrane integrity and stability to increase drought stress tolerance. Plant adaptation to drought stress involves significant changes in the activity of K^+ transport systems, both in root and leaf tissues. In shoots, regulation of GORK channels in stomatal guard cells is central in adaptation to drought and disruption of GORK activity resulted in impaired stomatal closure (Daszkowska-Golec and Szarejko, 2013).

1.2.3 Oxidative damage

Drought stress induces oxidative stress through the production of reactive oxygen species (ROS) such as superoxide (O_2^-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•), each with a characteristic half-life and an oxidizing potential. Plants produce ROS in chloroplasts, peroxisomes, mitochondria, endoplasmic reticulum, plasma membrane and the cell wall due to imbalance between generation and utilization of electrons under drought stress conditions (Tripathy and Oelmüller, 2012). In addition to organelles, plasma membrane together with apoplast is the main site for ROS generation in response to endogenous signals and exogenous environmental stimuli. Several types of enzymes, such as NADPH oxidases, amine oxidases, polyamine oxidases, oxalate oxidases, and a large family of class III peroxidases, that localized at the cell surface or apoplast are contributing to production of apoplastic ROS (Cosio and Dunand, 2009; Gill and Tuteja, 2010). Under drought stress condition, ROS production is enhanced through multiple pathways. A limitation in CO_2 uptake, caused by stress-induced stomatal closure, will reduce $NADP^+$ regeneration through the Calvin cycle that favors photorespiratory production of H_2O_2 in the peroxisome and production of superoxide and H_2O_2 or singlet oxygen by the over reduced photosynthetic electron transport chain (Noctor et al., 2014). An increased leakage of electrons to O_2 by Mehler reaction also occurs in PSI under drought stress (Shirao et al., 2013). It was shown that drought stressed wheat exhibited 50% increase in leakage of photosynthetic electrons to Mehler reaction as compared to wheat in control conditions (Biehler and Fock, 1996). An increase in the thylakoid membrane electron leakage to O_2 under drought stress was also observed in sunflower (Biehler and Fock, 1996; Sgherri et al., 1996). Under water stressed plants, ROS attack the most sensitive biological macromolecules in plant cells to induce lipid peroxidation, protein carbonylation, DNA damage and impair their functions to result in a catastrophic cascade of events and ultimately leads to death of the cells (Mishra et al., 2011; Sharma et al., 2012; Srivastava and Dubey, 2011) Fig 1.3.

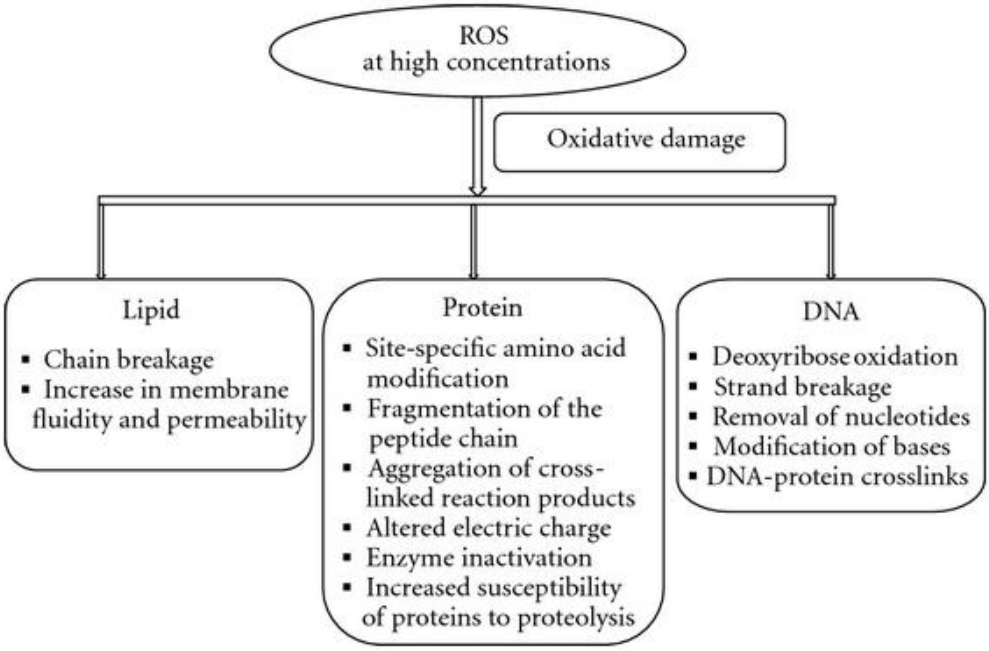

Figure 1.3 Reactive oxygen species (ROS) induced oxidative damage to lipids, proteins, and DNA. (Sharma et al., 2012)

1.3 Major mechanisms conferring drought tolerance

Drought resistance in plants is an extremely complex trait. When plants are confronted with drought, they respond to the stress by integrating an array of adaptive mechanisms at the anatomical, physiological and biochemical levels to enable them to sustain crop yield and development.

1.3.1 Anatomical features

Leaf traits

Leaf related traits involved in adaptive mechanisms towards drought stress conditions are leaf movements, leaf rolling and leaf size reduction. The leaves of many principle cereal crops such as sorghum, maize, rice and wheat roll (transverse rolling of the leaf lamina along the mid axis) when there is water stress condition and unroll for photosynthesis at the availability of water. This trait of leaves is identified as a pivotal trait during rainfed conditions, especially, if late rains occur at the time of grain-filling (Richards et al., 2001; Sirault et al., 2015). There are two different kinds of cells involved in leaf rolling (LR) in higher plants: bulliform and hypodermis cells. In

Graminae such as rice, maize, wheat and sorghum species, leaf rolling is caused by large, and highly vacuolated bulliform cells (motor cells) that occur in groups between vascular bundles on the adaxial epidermis of the lamina. Hypodermis cells are located under the epidermis. Huge hypodermis cells under the epidermis in *Ctenanthe setosa* involved in the modulation of leaf rolling. It has been shown that under drought conditions, these cells lose turgor pressure and shrink, leading to the rolling up of leaves. Once water is sufficient, these cells expand, and the leaves open again (Alvarez et al., 2008; Kadioglu and Terzi, 2007; Xiang et al., 2012). LR is an important and necessary mechanism protecting photosynthesis and reducing yield loss under drought stress by maintaining the leaf hydration, preventing loss of the photosynthetic pigments, sustaining the activity of PSII, keeping the stomata open, and conserving the activity of Rubisco (Kadioglu and Terzi, 2007; Kadioglu et al., 2012; Nar et al., 2009).

Leaf rolling (LR) might play a similar role in osmotic adjustment to maintaining internal plant water status. Late leaf rolling is the sign of turgor sustain despite of water stress, for instance via more water uptake or osmotic adjustment. LR also protects plants against the effects of excessive radiations (Kadioglu and Terzi, 2007; Subashri et al., 2009). Genes and proteins related to leaf rolling were identified in grasses under abiotic stress. (Luo et al., 2007; Yan et al., 2008; Zhu et al., 2017). In rice few genes involved in regulation of leaf rolling have been cloned. Two mutants with rolled leaves in rice designated as rl3(t)-1 and rl3(t)-2 were identified. Gene mapping result indicated that RL3 (t) gene resided in a 46-kb long region governed by the sequence tag site markers S3-39 and S3-36 on rice chromosome 3(Min et al., 2015). Another study revealed the isolation and characterization of SEMI-ROLLED LEAF1 (SRL1), a gene involved in the regulation of leaf rolling. Mutants srl1-1 (point mutation) and srl1-2 (transferred DNA insertion) exhibit adaxially rolled leaves due to the increased numbers of bulliform cells at the adaxial cell layers, which could be rescued by complementary expression of SRL1(Xiang et al., 2012).

The small, thick and evergreen leaf is another characteristic of adaptation to dry habitats in Mediterranean- climate vegetation. Drought stressed plants have significantly smaller leaf area than the control leaves (Casper et al., 2001; Gomez-del-Campo et al., 2003; Qing-cheng et al., 2003). Leaf size may decline owing to loss of turgor and overall resources limitation in stressful environments, making the construction of large leaves with extensive vascular and cell-wall fractions overly

expensive (Xu et al., 2009). This small and thick structured leaf appears to have both beneficial and detrimental aspects. The benefits are the ability to limit the water loss and to delay the onset of drought stress, while deleterious effects are related to a simultaneous restriction of CO_2 uptake (Arnon, 2012).

Root morphology

Plants constantly absorb water as well as nutrients from the soil through their roots. Consequently, the root system is generally recognized as a pivotal organ in accordance to improve crop adaptation to water scarcity. Some plants have strong capability to enhance root growth at the initial stage of water stress to obtain the water from deep soil (Hu and Xiong, 2014). Several studies have provided strong evidence towards two possibilities which are either root types penetrating deep into the soil and attaining greater root mass at depth (Ajithkumar and Panneerselvam, 2013; Ali et al., 2016; Lopes et al., 2011) or roots with large xylem diameters and larger lateral root systems with more root hairs are advantageous under drought conditions (Tanaka et al., 2014; Vadez, 2014). Such roots tend to have a greater total surface area, which facilitate maximal moisture and nutrient extraction to maintain photosynthesis (Blum, 2011; Comas et al., 2013).

A strong positive correlation has been observed between the penetration ability of roots and a drought tolerance index. The length, weight, volume, and density of plant roots were also identified to be linked with the drought tolerance in different field crops (Forster et al., 2005; Mohamed et al., 2002; Price et al., 2002). In dry areas, woody plant seedlings have vertical roots with tenfold difference to the length of the above ground height. With these substantial root traits, plants are capable to maintain a higher water potential and a longer duration of transpiration under water deficit, which provides additional advantages for their growth and development (Brunner et al., 2015; Larcher, 2003). The depth and range of soil moisture has an impact on rooting depth, volume and density. At the time of water shortage, plants vigorously adapt and alter their root structure by modifying their root growth in diverse manners depending on the species (Den Herder et al., 2010; Malamy, 2005). However, the deep water availability may improve crop growth and productivity either directly or through hydraulic redistribution, large diameter xylem vessels may be useful to enhance axial hydraulic conductivity of roots growing in deeper soil (Wasson, A. et al., 2012). Water stress tolerant genotypes have a tendency to change the partitioning of resources (dry

matter) towards the root (allometry) under water-limited stress conditions. This is showed as a higher root-to-shoot ratio either in terms of dry matter or length in field crops (Fenta et al., 2014; Fulda et al., 2011; Matsuo et al., 2013; Zhan et al., 2015). Since long, the root/shoot ratio has been used as a standard to depict the plant capability to drought tolerance. The standard in a shift of allometry supporting the roots (dry matter partitioning towards the roots) or potentially lessened lateral root expanding lies in the way that water stress tolerant genotypes balances resource allocation and use for every unit of water procured. Water stress tolerant genotypes hence decrease metabolic cost of soil water exploration to enhance the efficiency in obtaining of soil water under water stress (Matsuo et al., 2013).

A significant progress has been made in understanding the root traits and functioning in plant water acquisition, with several root QTL identified. In rice a total of 24 regions were identified as containing QTLs (these regions often contained several QTLs identified for different root traits). In rice the *DRO1* gene on chromosome 9 have been identified and cloned which is associated with rooting depth. After backcross introgression of this gene into the IR64 variety of rice an increase in drought tolerance was seen in drought environments with no apparent reduction in grain yield under well-watered conditions (Price et al., 2002; Uga et al., 2013). A total of 15 QTL effects, 6 additive and 9 epistatic, were detected for different traits of root length and root weight in 1RS wheat (Sharma et al., 2011). In the model plant *Arabidopsis thaliana*, researchers have identified QTL for ABA induced reduction in the lateral root growth as well as root system plasticity and size (Gerald et al., 2006; Xiong et al., 2006). In maize, a major constitutive QTL, designated *Root-ABA1*, was associated with the crown root branching, diameter, and angle, as well as whole root dry mass. Moreover, increases in water uptake have also been associated with the up-regulation of aquaporin genes *PIP1* and *RWC-3* in maize, which shows that root physiology, in additional to or concurrent with, shifts in root system size, can be associated with increased capacity of root systems to acquire water (Giuliani et al., 2003; Giuliani et al., 2005).

Cuticle structure and trichomes

Higher plants have developed an extracellular hydrophobic cuticular layer that covers their aerial organs including leaves, flowers, fruits and young stems that provides protection against several stress factors including water stress and restrict transpiration. Cuticle is a heterogeneous layer consisting of cutin polyester matrix covered with epicuticular waxes and filled with intracuticular waxes. Cuticular waxes, mainly composed of very-long-chain alkanes, fatty acids, primary and secondary alcohols, esters, aldehydes, and ketones, are responsible for the glossy appearances in leaves and fruits (Bernard and Joubès, 2013; Yeats and Rose, 2013).The thickness of this membrane vary from 0.05μm in some mesophytic plants to as thick as 225μm in xerophytic species (Fernandez et al., 2016; Goodwin and Jenks, 2005). Most angiosperms and gymnosperms have a restricted capability to resist dehydration. For example, irreversible leaf damage appears in most plants if, roughly, half of the water content is lost (Burghardt and Riederer, 2003). To prevent this, plants close their stomata when internal water content lessens down under a certain threshold. However, even after stomatal closure, plant continues to lose water through the cuticle, albeit at a much lower late. Consequently, the plant dies if water-limiting conditions persist for a long time. It is the period after drought-induced stomatal closure when the water loss is most affected by the cuticle resistance to water vapour flux and when the cuticle becomes pivotal (Goodwin and Jenks, 2005). The mechanical structure and a chemical composition of cuticle lipids vary considerably between plant species, and in response to environmental stimuli and stresses. Several studies have indicated that drought can induce increased wax deposition on the leaf surfaces of different plant species, including Arabidopsis (Yang et al., 2011), peanut (Samdur et al., 2003), and tree tobacco (Cameron et al., 2006).

Thickened cuticles not only reduce water loss by evaporation but also prevent leaf damage and breakage under wilting. Some plants have shiny cuticles, which reflect light and reduce the heat load of the leaves. Cuticle-related transcriptional factors have been identified and characterized for their regulation of cuticle-associated genes in a number of plant species, including Arabidopsis, rice, barley, maize, Medicago, soybean, tomato and wheat (Bi et al., 2017; Borisjuk et al., 2014; Buxdorf et al., 2014; Giménez et al., 2015; La Rocca et al., 2015; Sela et al., 2013; Xu et al., 2016).

The increased number of trichomes may play a role in water retention by trapping a layer of damp air in the microclimate of the guard cells, limiting transpirational water loss when the stomata open for CO_2 uptake (Taiz and Zeiger, 2010). This air trapping function is probably more sufficient in species with hairs concentrated in recessed stomata (Smith et al., 2009). Trichomes are also important because they reflect light, thus reducing leaf temperatures and so transpiration rates. Similarly, for adapting to limited water availability, vascular epiphytes utilize their thickened cuticles and stomata surrounded by trichomes. The leaf cuticles of vascular epiphytes may act as efficient barriers against water loss after their stomata close (Franco Pinheiro et al., 2013; Helbsing et al., 2000).

Succulency

Possession of succulent organs such as leaves, stems or roots alleviate the impact caused by drought stress. Among different plants, succulent organs that appear outwardly similar can in fact reserve water in different tissues. For instance, in cacti with tuberous roots, water is stored in woody tissues (Stone-Palmquist and Mauseth, 2002). Contrary to this, closely related *G. bracteata* (Anacampserotaceae) stores water in the expanded root cortical tissues (Eggli and Nyffeler, 2009). The succulence starts at the cellular level by the development of large central vacuole, capable to store sufficient water and other substances. This modification facilitates water homeostasis and buffering the plant water stress condition. As the stomata of succulents open during night, therefore these plants fix carbon eventually during the day with the help of reserved carbon dioxide in the form of malic acid and CO_2 discharged internally by respiration (Becker, 2007; Ogburn and Edwards, 2010). To augment water storage, leaf succulence is constantly linked with the use of some types of photosynthetic Crassulacean acid metabolism (CAM), including obligate CAM, CAM-idling, CAM-cycling, and flexible CAM systems. CAM and leaf succulence may have some mechanistic links or may have evolved separately to maximize water use strategies in arid environments. The recommended reliance of the leaf succulence to CAM comes from two considerations. The first one is the reliance on CAM, which needs the extra storage ability for the C_4 acids given by the extended succulent cells linked with high vacuolar volumes (up to 98% of cell volume). Secondly, as leaf succulence augments, the mesophyll conductance for the diffusion of CO_2 through hydrated succulent leaf tissue of the photosynthetic tissue lessens down. (Herrera, 2009; Nelson et al., 2005; Ogburn and Edwards, 2010; Tomás et al., 2013). That is why CAM plants can achieve

higher water use efficiencies that are three to six-fold greater than C_4 and C_3 species respectively (Becker, 2007; Ogburn and Edwards, 2010; Yamori et al., 2014). In addition to the leaf succulency, Crassulacean acid metabolism plants also possessed sunken stomata and less stomatal density that help them prevent excessive water loss and irradiation (Lledías et al., 2017).

1.3.2 Physiological mechanisms

Root traits

Adjustment of water uptake to soil-water availability through modifications in physiology of roots is crucial for adaptation to water stress conditions. It depends on soil, soil-root air gaps, and root hydraulic conductivity. Researchers consistently highlighted a decrease of root hydraulic conductivity under water stress conditions (Bao et al., 2014). Similarly, the desert succulents are able to survive prolonged drought by stopping hydraulic water flow through large accumulations of suberin in the exodermal and endodermal cells; this mechanism permits gradual exploitation of residual soil moisture (North and Nobel, 1998). Contribution of aquaporins acting as channels for the passive water flow across root membranes under water stress is promising for development of water resistance. Biosynthesis and transport of auxin (major membrane inherent proteins) required in lateral root synthesis, are activated in response to contact with water, giving a connection between the external environmental signal and activation of lateral root advancement more deeper inside the root (Aroca et al., 2006; Bao et al., 2014; Gao et al., 2010; Trifilò et al., 2004). The response of AQPs to water stress can lead to up- or down-regulation of gene expression or even no change depending on the duration and intensity of the stress (Galmés et al., 2007; Maurel et al., 2010). Other putative physiological root traits with potential to confer tolerance in various crops include osmotic adjustment. Osmotic adjustment is achieved by either increased uptake of inorganic ions (mainly K^+, Na^+ and Cl^-) or by accumulation of compatible solutes such as free amino acids, proline and sugars in the roots (Chen and Jiang, 2010). The role of accumulation of these compatible solutes in root elongating zone is to maintain turgor pressure to continue root elongation and root growth in drying soils, which enable the plant to maintain its transpiration by exploiting a greater volume of soil or utilize available water in a given soil volume more efficiently (Zhang et al., 2017). Under water stress conditions, osmotic adjustment usually commenced earlier in roots than in shoot to maintain root growth

and the absorption of water and nutrients, and thus to delay the occurrence of water deficit in the shoot (Ogawa and Yamauchi, 2006). Different studies have proved the significant accumulation of compatible solutes in roots such as proline, sugars and amino acids under water deficit conditions (Devi and Sujatha, 2014; Ogawa and Yamauchi, 2006; Velazquez-Marquez et al., 2015). Inorganic ions including K^+, Na^+ and Cl^- also make significant contribution towards osmotic adjustment (Chen and Jiang, 2010). There are several approaches to explore ion relations at root tissue and cellular levels, e.g., flame photometry, ion chromatography and X-ray microanalysis, however, these traditional methods give us a concentration of elements that generally presents static information (Sun et al., 2009). The non-invasive ion flux techniques (SIET and MIFE) are a powerful tool to investigate the dynamic flux of ions which enable us to understand the detailed mechanisms of how plants control ion homeostasis in roots under abiotic stresses including osmotic stress (Kunkal et al., 2006; Shabala, 2006; Shabala and Lew, 2002).

Stomatal regulations

Stomata opening and closing is accomplished by the swelling and shrinkage of the guard cells, which is driven by ion exchange; the cytoskeleton reorganization and metabolite production; the regulation of gene expression and the posttranslational change of proteins (Kim et al., 2010). Swelling of the guard cells brings about stomata opening since the content of ions and osmolytes inside them makes them larger and subsequently move away from each other making the stomatal aperture bigger. In contrast, closing is an inverse mechanism and results in the contracting of the guard cells when the efflux of ions occurs. The guard cell turgor is dynamically acclimated to environmental conditions and hormonal signals in order to aid the proper gas exchange and prevents extreme water loss. Mature guard cells do not possess plasmodesmata and consequently most influx and efflux of solutes occurs by means of ion channels, transporters, and pumps that are confined in the plasma membrane (PM) (Daszkowska-Golec and Szarejko, 2013)

Signaling Pathways

The source and function of abscisic acid

Under water stress, ABA is the central signaling phytohormone responsible for the regulation of important processes such as stomatal closure (Jiang and Hartung, 2008). One of the main reactions perceived after the water deficit is ABA accumulation in plant tissues. ABA synthesis is proposed to occur in various plant organs, for example, leaves and roots, additionally in vascular tissues, and it moves to target cells through both xylem and phloem, permitting a two-way transportation amongst roots and shoots. The diminishing soil water potential is perceived by roots, activating ABA synthesis as a reaction to lessening root water potential (Luo et al., 2014; Puertolas et al., 2013) which is then transported through xylem sap to leaves. ABA is synthesized in both roots and leaves (Correia et al., 2014; Soar et al., 2006; Thompson et al., 2007). The significance and the role of the root-sourced ABA is still questionable. Various studies have shown that ABA can be produced in large number at a prior stage in leaves relative to roots in response of drought (Ikegami et al., 2009). Also, some reports suggest that ABA-induced stomatal closure is not subject to ABA release from roots (Christmann et al., 2007). During water deficit, dissimilar to roots (Ernst et al., 2010), leaves show the full suite of ABA-biosynthetic genes in vasculature (Okamoto et al., 2009) and possess the capacity to transport this ABA to the site of activity, the stomata (Kuromori et al., 2014). Grafting experiments have been utilized to find the source of abscisic acid in water stress-induced stomatal closure. Some of these trials propose that leaf-sourced ABA is pivotal for stomatal closure and foliage-derived ABA is promptly transported to the roots where it is crucial for regulating normal root ABA levels (McAdam et al., 2016).

ABA accumulation in guard cells

The increase in ABA concentration in guard cells is trigged by the reduction in the amount of water around the roots. Christmann et al. (2005) demonstrated a basic time course for drought-induced ABA accumulation in tissues of Arabidopsis seedlings whose roots were subjected to a -1.0 MPa water stress treatment. An increase in ABA concentration in the vascular tissue of the cotyledons was observed within 4 h after treatment. After 4 h, ABA was relatively uniformly distributed in the leaf tissue, but by 8 h post treatment, a higher concentration of ABA was present in guard cells than in other leaf tissue. From the vascular tissue, a specific type of ATP-binding cassette

transporter exports ABA, while another transporter imports ABA into leaf tissue, including guard cells (Kuromori et al., 2010; Umezawa et al., 2010).

How ABA triggers signaling pathways

The ABA signaling network that leads to stomatal closure under drought stress is activated by the perception of ABA. The earliest events occur via a central signaling module made up of proteins belonging to three protein classes: Pyracbactin Resistance/Pyracbactin resistance-like/Regulatory Component of ABA Receptor (PYR/PYL/RCARs) proposed to be the ABA receptors, Protein Phosphatase 2Cs (PP2Cs) which act as negative regulators, and SNF1-related protein kinase 2s (SnRKs) which are positive regulators. In the presence of ABA (Fig 1.4B), the PYR/PYL/RCAR-PP2C complex formation leads to inhibition of PP2C activity, thus allowing activation of SnRKs, which target membrane proteins, ion channels and transcription factors, and facilitate transcription of ABA-responsive genes. In the absence of ABA (Fig 1.4A), PP2Cs negatively regulate activation of SnRK2 kinases. Without activation of SnRK2, downstream ABA signaling targets are inactive (Cutler et al., 2010; Harrison, 2012; Hubbard et al., 2010).

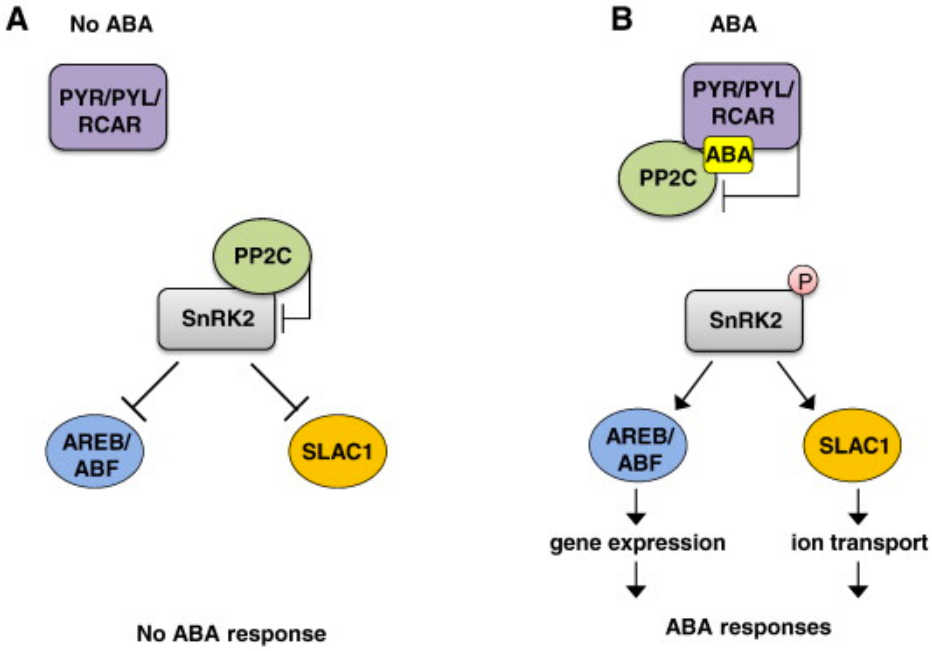

Figure 1.4 The schematic representation of major ABA signaling pathway in plants with or without ABA presence (Sah et al., 2016).

Regulation of ion channels at the time of stomatal closure

The activated SnRK2 acts as a positive regulator of ion channels on the plasma membrane, including the anion exporter slow anion channel-associated 1 (SLAC1) and the K^+ channel in Arabidopsis thaliana (KAT1) in guard cells (Hubbard et al., 2010). SnRK2 activates SLAC1 through phosphorylation, while phosphorylation of KAT1 by SnRK2 inhibits its function, thus decreasing the influx of K^+ into the cell. Increased SLAC1 activity causes an efflux of anions, which depolarizes the membrane and results in the loss of K^+ through the depolarization-activated K^+ efflux channel called guard cell outward-rectifying K^+ (GORK) (Daszkowska-Golec and Szarejko, 2013) (Fig 1.5B). The collective loss of anions and K^+ ions from the guard cells causes water to move out of these cells, which results in the reduction in turgor that triggers stomatal closure in response to ABA. The elevation of the Ca^{2+} concentration as a result of the Ca^{2+} release via channels in the plasma membrane and tonoplast is also an important event facilitating stomatal closure (Hosy et al., 2003; Jeanguenin et al., 2008).

Regulation of ion channels at the time of stomatal opening

During the opening of the stomata, a depletion of endogenous ABA is observed through xanthophyll cycling, the isomerization of ABA precursors and the activation of ABA catabolism enzymes, such as CYP450 (cytochrome P450). The degradation of ABA liberates the guard cells to extrude H^+ via H^+-ATPase pump. The efflux of H^+ hyperpolarizes the plasma membrane and leads to K^+ uptake via activation of inward K^+ rectifying channels, such as KAT1 (potassium channel in *Arabidopsis thaliana* 1), KAT2 (potassium channel in *Arabidopsis thaliana* 2), and AKT1 (*Arabidopsis thaliana* K^+ transporter 1) (Pilot et al., 2001; Ueno et al., 2005).

An additional signal that triggers the influx of K^+ through K^+ channels is the acidification of the apoplast as a result of H^+ extrusion from the guard cells. K^+ uptake is adjusted by counter-ions, especially Cl^- acquired from the apoplast, and $malate^{2-}$ that is retrieved from the starch breakdown. The last one is transported from the apoplast with the help of a nitrate transporter $AtNRT_{1.1}$. The essentiality of NO_3^- uptake was determined by an analysis of Arabidopsis clh1 mutant. The stomatal apertures of the *chl1* mutant were relatively smaller than those of the wild-type when nitrate was provided. Also, the *chl1*mutant was drought tolerant (Guo et al., 2003). Ions supplied to the guard cells together with water transferred through aquaporins

create the turgor that are important to keep stomata open (Fig 1.5A) (Daszkowska-Golec and Szarejko, 2013).

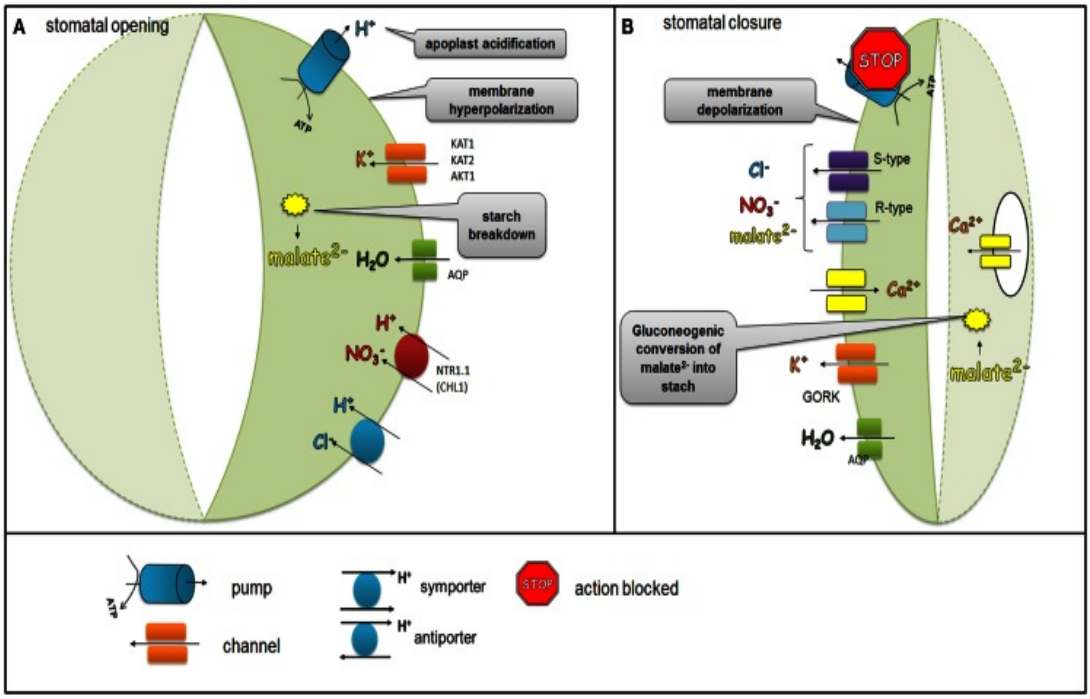

Figure 1.5 Modulation of ion channels, pumps, and transporters confined in the plasma membrane of the guard cells during stomatal opening and closure. Adopted from Daszkowska-Golec and Szarejko (2013).

ABA induces calcium signaling pathways

The plant cells possess various compartments such as vacuole and endoplasmic reticulum, which stores Ca^{2+} that can be discharged into the cytoplasm when required. Particular channels/pumps control the movement of Ca^{2+} in and out of cells and organelles (Mahajan et al., 2006; Tuteja and Mahajan, 2007). Ca^{2+} release can be primarily from the extracellular source (apoplastic space) as inclusion of EGTA or BAPTA was evident in different cases to block calcium effects. Ca^{2+} release as a consequence of activation of PLC (Phospholipase C), leading to hydrolysis of PIP2 to IP3 and subsequent release of Ca^{2+} from intracellular Ca^{2+} stores (Tuteja and Sopory, 2008a). Numerous Ca^{2+} dependent protein kinases (CDPKs) are activated during drought stress conditions and control stomatal closure through regulation of ion channels. In ABA-associated regulation of slow anion channel-associated 1(SLAC1), SnRK2 inhibits the phosphatase ABA insensitive 1(ABI1). This ABI1 inactivation allows the activation of CPK21 (a CDPK) which phosphorylates SLAC1, and activates anion efflux i.e. Cl^-, NO_3^- and $malate^{2-}$(Geiger et al., 2010). In Arabidopsis leaves, the concentration of another CDPK, CPK10, increases within 30 min after drought stress

begins and causes the inhibition of inward K+ currents (Zou et al., 2010). These results indicate that an increase in the cytoplasmic concentration of Ca^{2+} stimulates Ca^{2+}-dependent pathways that inhibit K^+ import while activating SLAC1, triggering the membrane depolarization that activates K^+ efflux. CDPK pathways thus contribute to the loss of ions from guard cells, which in turn result in the loss of turgor, and ultimately to the stomatal closure.

1.3.3 Biochemical mechanisms

Osmoprotectants

Plants tend to cope with water deficit stress by a process known as osmotic adjustment. During this process, plants decrease their cellular osmotic potential by the accumulation of solute. Plants synthesize and accumulate large number of osmotically active compounds or osmoprotectants in the cytosol, which play a key role in maintaining cell turgor (Farooq et al., 2008; Filippou et al., 2014; Li et al., 2012; Parida et al., 2008). Osmoprotectants or compatible solutes are small molecules having low molecular weight, electrically neutral, highly soluble and non-toxic at molar concentrations. They help plants to survive in extreme osmotic environment (Ahn et al., 2011; Lang, 2007). At the same time, these compatible solutes or osmoprotectants can stabilize proteins and membranes, and reduce the osmotic potential of membranes to prevent dehydration inside the cell and keep the cells in dehydrated state (Hussain Wani et al., 2013; Munns and Tester, 2008). They are comprised of proline, sucrose, polyols, trehalose as well as quaternary ammonium compounds (QACs) including glycine betaine, alanine betaine, proline betaine, choline-O-sulfate, hydroxyprolinebetaine, and pipecolatebetaine. The hydroxyl group of sugar alcohols substitutes the OH group of water to maintain the hydrophilic interactions with the membrane lipids and proteins. Thus, these molecules help to maintain the structural integrity of the membranes (Mahajan and Tuteja, 2005; Ramanjulu and Bartels, 2002). Osmoprotectants can be categorized into four groups:

- Ammonium compound groups (Betains, polyamines and related compounds)
- Carbohydrate sugars (Glucose, Sucrose, Fructose, Fructan and Trehalose)
- Sugar alcohols (Mannitol, Sorbitol and D-Ononitol)
- Amino acids (Proline)

1. Ammonium compounds

Betaines, belonging to quaternary ammonium compounds group including glycine betaines (GB), b-alaninebetaine, prolinebetaine, choline-O-sulphate, dimethyl sulphoniopropionate, hydroxyproline betaine, and pipecolate betaine, act as effective compatible solutes. In response to several environmental stresses (including drought), GB accumulates in chloroplasts of some plants. At the same time, glycine betaines facilitate water flow into cells for regulating the intracellular osmotic equilibrium and maintains the cascade of signal transduction under stress condition. The key role of osmoprotectants is to maintain turgescent cells and regulate the water potential in order to ensure impartial water relation (Ashraf and Foolad, 2007; Wang et al., 2003).

In glycine-betaine biosynthetic pathway, GB is formed during two successive oxidation processes of choline via choline monooxygenase (CMO) and betaine aldehyde dehydrogenase, and whole reactions are performed in chloroplasts especially in stroma and catalyzed by CMO and NAD dependent betaine aldehyde dehydrogenase (BADH) enzymes (Hussain Wani et al., 2013) (Fig 1.6).

Extensive research has been focused on the accumulation of betaines in various crops under drought stress (Jagendorf and Takabe, 2001; Sawahel, 2003). In a same manner exogenous application of GB enhance its level internally and may enhance plant growth and crop yield of various plants under water deficit (Rezaei et al., 2012).

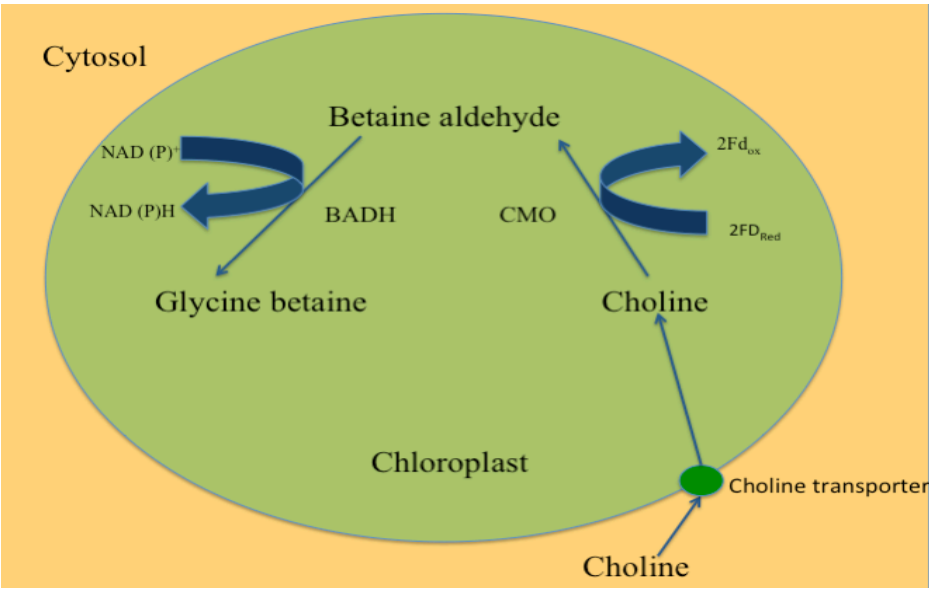

Figure 1.6 Glycine-betaine biosynthesis pathway, Adopted from Ahmad and Sharma (2008)

2. Carbohydrate sugars

Carbohydrates are the principal product of photosynthesis and also the essential form in which carbon is transported and partitioned within the plant. Since water stress effects photosynthesis, variations in the levels of carbohydrates including starch and soluble sugars often occur under drought stress conditions. Evidences show that higher accumulation of reduced form of sugars such as glucose, sucrose, fructose and fructans functions as osmoprotectants under drought stress (Chołuj et al., 2008; Hoffmann, 2010). Trehalose is a non-reducing sugar and contribute as a source of energy and carbon and as a protective molecule against drought. It can stabilize proteins and membranes of plants when exposed to stress (Iturriaga et al., 2009; Redillas et al., 2012). The sugars sucrose and raffinose are also known to protect cells against oxidative damage and accumulate later during responses to stress (Cramer et al., 2007).

3. Sugar alcohols

Sugar alcohols (often called polyols) may be defined in two ways on the basis of their structure. The first one is cyclic structure consisting of myo-inositol and pinitol, and another one is linear structure including sorbitol, mannitol, xylitol and ribitol (Singh et al., 2015). Polyols are non-reducing, water-soluble sugar alcohols that are generally formed through the reduction of aldoses or their phosphate esters. Unlike myo-inositol, sorbitol and mannitol are direct products of photosynthesis, particularly in fully expanded leaves (Noiraud et al., 2001; Tari et al., 2010). Polyols normally accumulate in the cytosol and counteract the adverse impacts of multifarious stresses on metabolism. In addition to osmoregulation, polyols help plants in ROS detoxification, protection of membrane integrity and enzymes/protein stabilization under drought stress (Ashraf and Foolad, 2007; Ashraf and Harris, 2004). Enhanced transport of polyols has been observed in both xylem and phloem in response to water stress (Noiraud et al., 2001).

4. Amino acids

Amino acid proline is another important osmoprotectant contributing to osmotic adjustment. Progressive drought stress induces a significant accumulation of proline in the cytoplasm of many plant species in response to many environmental stresses, and it plays a significant role in reducing the adverse effects of stress with varying degrees in different plant species (Ashraf and Foolad, 2007; Hsu et al., 2003; Kishor

et al., 2005). Proline can also act as a signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for plant recovery from stresses (Szabados and Savoure, 2010). In plants, proline biosynthesis occurs in the cytosol while its degradation takes place in the mitochondria (Ashraf and Foolad, 2007). In this process of biosynthesis there are two different precursors i.e., glutamate and ornithine and pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5- carboxylate reductase (P5CR) are the two key enzymes of this biosynthetic pathway (Nounjan et al., 2012). The conversion of glutamate to Pro takes place by two consecutive steps, first is catalyzed by P5CS, which is bifunctional enzyme (Fig 1.7). It catalyzes activation of glutamate by phosphorylation and second is P5CR activity which reduces intermediate c-glutamyl phosphate into glutamate semialdehyde (GSA), both these functions involved in Pro biosynthesis and catabolism (Verbruggen and Hermans, 2008). In a same way, ornithine enzyme occurs in mitochondria can be transmitted to P5C through the action of Orn-d-aminotransferase (OAT). Moreover, another pathway of proline biosynthesis is gluthione via the action of glutamic-g-semialdehyde (GSA) and D1-pyrroline-5-carboxylate (P5C). The P5C synthase (P5CS) enzymes catalyses conversion of glutathione to P5C, followed by the action of P5CR enzyme which reduces the P5C to proline (Parvaiz and Satyawati, 2008; Singh et al., 2015) (Fig 1.7).

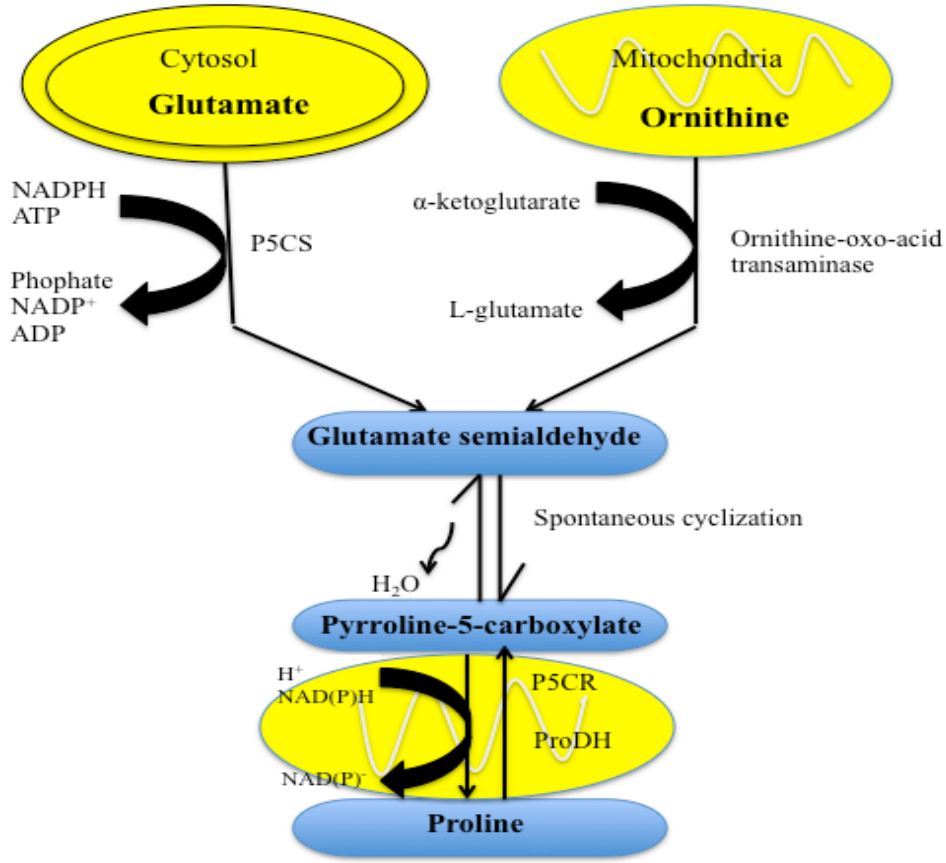

Figure 1.7 Biosynthetic pathway of proline through glutamate and ornithine in plants. P5CS (pyrroline-5-carboxilate synthetase), P5CR (pyrroline-5-carboxilate reductase), ProDH (proline dehydrogenase). Adopted from Parvaiz and Satyawati (2008)

1.3.4 Reactive oxygen species (ROS)

The generation of reactive oxygen species (ROS) is one of the earliest biochemical responses of plant cells under abiotic stresses (Lee et al., 2012); these ROS act as secondary messengers to trigger subsequent defense reactions (Farooq et al., 2009b). ROS seem to have a dual effect under abiotic stress conditions that depend on their overall cellular amount. If kept at relatively low levels they are likely to function as components of a stress-signaling pathway, triggering stress defense/acclimation responses. However, when the level of ROS exceeds the capacity of defense mechanisms, the cells are in the state of oxidative stress. Plants have evolved efficient enzymatic and non-enzymatic antioxidative systems to protect themselves against oxidative damage, and fine modulation of low levels of ROS for the signal transduction. ROS-scavenging enzymes of plants include enzymatic enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) and some other non-enzymatic antioxidants like

glutathione (GSH), ascorbate (ASC) and carotenoids, flavonoids, α-tocopherol and osmolyte proline (Table 1.2) (Gill and Tuteja, 2010; Kubiś et al., 2014; Noctor et al., 2014).

Efficient destruction of O_2^- and H_2O_2 in plant cells require the concerted action of antioxidants, O^-_2 can be dismutated into H_2O_2 by SOD in the chloroplast, mitochondrian, cytoplasm and peroxisome. POD plays a key role in scavenging H_2O_2, which was produced through dismutation of O_2^- catalyzed by SOD. CAT is the main enzyme to eliminate H_2O_2 in the mitochondrion and peroxisomes and thus help in ameliorating the detrimental effects of oxidative stress (Sofo et al., 2015). Maintaining a high level of antioxidative enzyme activities and the capability of antioxidant enzymes to scavenge ROS has been found to be correlated with drought tolerance in plants (Sharma and Dubey, 2005). Details of all enzymatic and non-enzymatic antioxidants including their locations and functions are given below in Table 1.2. Modifications in the activity of antioxidant enzymes can be used to predict drought tolerance in plants. It was evaluated that when most of the enzymes activities are upregulated under water stress conditions, the plants were likely to be drought tolerant. The study concluded the status of antioxidant enzymes could provide a meaningful tool for predicting drought (Devi et al., 2012).

Table 1.2 List of enzymatic and non-enzymatic antioxidants with their function and location

Enzymatic antioxidants	Enzymatic code	Reaction catalyzed	Subcellular location
Superoxide dismutase(SOD)	1.15.1.1	$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow 2H_2O_2 + O_2$	peroxisomes, mitochondria, cytosol, and chloroplast
Catalase (CAT)	1.11.1.6	$2H_2O_2 \rightarrow O_2 + 2H_2O$	peroxisomes and mitochondria
Ascorbate peroxidase (APX)	1.11.1.11	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	peroxisomes, mitochondria, cytosol, and chloroplast
Monodehydroascorbate reductase (MDHAR)	1.6.5.4	$2MDHA + NADH \rightarrow 2AA + NAD$	mitochondria, cytoplasm, and chloroplast
Dehydroascorbate reductase (DHAR)	1.8.5.1	$DHA + 2GSH \rightarrow AA + GSSG$	mitochondria, cytoplasm, and chloroplast
Glutathione reductase (GR)	1.6.4.2	$GSSG + NADPH \rightarrow 2GSH + NADP^+$	mitochondria, cytoplasm, and chloroplast
Guaiacol peroxidase (GPX)	1.11.1.7	$H_2O_2 + DHA \rightarrow 2H_2O + GSSG$	Mitochondria, cytoplasm, and chloroplast, and ER
Non-enzymatic Antioxidants	Function		Subcellular location
Ascorbic Acid (AA)	Detoxifies H_2O_2 via action of APX		cytosol, chloroplast, mitochondria, peroxisome, vacuole, and apoplast
Reduced Glutathione (GSH)	Acts as a detoxifying co-substrate for enzymes like peroxidases, GR and GST		cytosol, chloroplast, mitochondria, peroxisome, vacuole, and apoplast
α - Tocopherol	Guards against and detoxifies products of membrane LPO		Mostly in membranes
Caroteniods	Quenches excess energy from the photosystems, LHCs		chloroplasts and other non-green plastids
Flavonoids	Direct scavengers of H_2O_2 and $^1O_2$ and $OH^{\bullet}$		Vacuole
Proline	Efficient scavenger of $OH^{\bullet}$ and $^1O_2$ and prevent damages due to LPO		Mitochondria, cytosol, and chloroplast

1.4 Screening for drought tolerance

Plant breeders are in a need for convenient, reproducible, reliable, and rapid screening methods to be used as a proxy for drought tolerance for a large number of genotypes. Field screening has long been suggested as the ultimate proof for drought tolerance because of providing real and natural drought conditions for plants and a large germplasm can be evaluated in field conditions with no limitations of space (Ghulam Rabbani et al., 2015; Neelam et al., 2018; Zou et al., 2007). However, the reliability of screening is somewhat compromised due to variation in weather conditions throughout the growing season and from year to year (Longenberger et al., 2006).

As yield is the prime goal of the plant breeders, it is hardly surprising that most of the studies have been focussed on yield and yield related traits in different crops under drought stress (Al-Abdallat et al., 2017; Kilic and Yagbasanlar, 2010; Sakai et al., 2010). However, measuring yield is a time consuming and labour-intensive process because it requires the screening trial to last through the entire plant ontogeny (Szira et al., 2008). Recent imaging techniques to estimate biomass alleviated the issues generated in traditional methods of measuring yield and destruction of harvested plants to some extent but there is still loss of reliability and accuracy because plants grow larger and produce multiple shoots (Munns et al., 2010).

Unlike field experiments, drought stress treatments imposed during vegetative growth stage under glasshouse conditions are less costly, and the intensity of the stress applied may be easily monitored (Chen et al., 2016; Schiop et al., 2015). In most cases, plants are grown in small pots because of the limited space in a glasshouse, and also because the soil drying is faster. However, the use of small pots could have a drawback that a smaller pot implies a small amount of soil and thereby, almost invariably, a reduction in the availability of water and nutrients to the plant as compared to field conditions (Poorter et al., 2012). Researchers working with small pots also considered the fact that completely stopping irrigation caused rapid drying and might prevented the plant from adjusting to the new conditions. This, of course, is different from field conditions, under which plants are more gradually exposed to water deficits (Negin and Moshelion, 2017).

One crucial point to be considered in screening is that different drought tolerance mechanisms are required in different growth stage or different drought conditions.

Effects of drought stress on vegetative and reproductive processes would cause yield reduction in different manners (Blum, 1996; Sanchez et al., 2012). Therefore, physiological traits needed for tolerance in each growth stage is also different. In addition, tolerance mechanisms are expected to be different depending on drought severity. This was highlighted by a study by Clauw et al. (2015b) where the expressed gene sets were largely different between *Arabidopsis* plants under mild and severe drought conditions. Therefore, it has been proposed to screen plants separately in different growth stage and drought conditions to characterize drought tolerance (Iseki et al., 2018). Another important concern of the plant breeders is of whether the screening for drought tolerance should be done in optimum conditions or targeted drought conditions. While some researchers have proposed selection under non-stress condition (Betran et al., 2003; Rajaram and van Ginkel, 2001), others have focused on selection under target stress conditions (Mohseni et al., 2016). A "hybrid approaches" involving selection under both non-stress and stress conditions have been also advocated (Gholinezhad et al., 2014; Sharafi et al., 2011).

Assessing different physiological traits have been proposed as suitable proxies for measuring drought tolerance. The measurements of most of the physiological characteristics are rapid, less laborious and non-invasive compared with agronomical traits and therefore are preferred for large scale screening operations. Leaf transpiration has been suggested as a direct measure of plant water consumption. However, with the increase of stress severity, leaves are often rolled making this unfeasible to measure (Hasanuzzaman et al., 2017). Similarly, SPAD values are usually used as rapid and cost-effective assessments of drought tolerance (Filek, M et al., 2015; Sharma et al., 2015). However, the results may be somewhat misleading as although drought stress negatively affects chlorophyll biosynthesis, chlorophyll density per unit of area may increase as a result of reduced leaf growth and thicker leaves in stressed plants (Rao and Wright, 1994). Nonetheless, there are many studies reporting decrease in SPAD values under drought stress conditions (İstipliler et al., 2016; Naderikharaji et al., 2008; ÖZTÜRK and Aydin, 2017).

1.5 Unanswered questions and aims of this study

As shown above, drought stress drastically inhibits plant cellular mechanisms and impairs plant growth and productivity. Improving drought resistance in plants is a challenge for plant breeders and crop physiologists. Plants have evolved complex morphological, physiological and biochemical adaptations in response to drought stress conditions. These adaptive mechanisms include mainly stomatal regulations, root traits and ormoregulations. Root length is linked with extraction of water and nutrients from the deeper layers therefore it could be used as the most beneficial trait measuring water stress tolerance. However, longer root length comes with high carbon cost, therefore the essentiality of this trait for maintaining plant growth and productivity under drought stress remains to be validated. Under water deficit, plants close their stomata to limit water loss, consequently decreasing photosynthesis via reduced entry of CO_2. Some previous studies showed that to cope with drought stress conditions, stomata can adjust their aperture by opening to optimize CO_2 influx and closing to minimize transpiration rates. It is still unclear whether regulation of stomata is attributed to stomatal density and stomatal aperture. Drought-induced osmotic stress requires osmotic adjustment either by the uptake of nutrients or by the *de novo* synthesis of compatible solutes. However, their relative contribution is still disputed in the literature. Moreover, less attention has been given to the role of Cl- in the osmotic adjustment compared to the effects of K and Na as Cl is considered to be a toxic element because of its high concentrations in the soil. There is still ambiguity of whether Cl has a substantial role with K and Na or whether it does not contribute significantly toward the osmotic adjustment. Two major obstacles in the progress of plant breeding are the lack of reliable and convenient screening technique to measure drought stress tolerance and the choice of the appropriate physiological traits to be targeted as a proxy for drought tolerance. Addressing these issues, broad range of barley and wheat genotypes will be used in this study for revealing the genotypic variability, assessing the suitability of different screening methods and providing a comprehensive understanding of physiological mechanisms underlying drought tolerance. Moreover, this study will also reveal mechanisms of osmoregulation and abscisic acid-mediated signaling in hyperosmotically stressed barley roots.

1.6 Outline of the chapters

Chapter 2: Materials and Methods of four sets of experiments conducted in this study

Chapter 3: Assessing the extent of genetic variability in drought tolerance in wild and cultivated barley germplasm

Chapter 4: Genotypic variation of drought tolerance in bread and durum wheat

Chapter 5: Revealing key physiological traits conferring drought stress tolerance in barley

Chapter 6: Revealing mechanisms of osmoregulation and abscisic acid- mediated signaling in hyperosmotically-stressed barley roots

Chapter 7: General discussion and future research

Chapter 2. Materials and Methods

2.1 Glasshouse Experiments

2.1.1 Plant material

Thirty varieties of barley (*Hordeum vulgare* L.), 18 bread wheat (*Triticum aestivum* L.) and two durum wheat (*Triticum durum*) varieties were selected for the present study. All the seeds were obtained from the Australian Winter Cereal Collection and multiplied at the Launceston facilities of the Tasmanian Institute of Agriculture (TIA). A list of the varieties and their origin is given in Table 2.1 & 2.2

Table 2.1 Selected barley varieties and their origin

Wild Barley	Origin	Cultivated Barley	Origin
X115	Tibet, China	Clipper	Australia
X030	Tibet, China	CM72	USA
X112	Tibet, China	Commander	Australia
X165	Tibet, China	Flagship	Australia
X117	Tibet, China	Franklin	Australia
X045	Tibet, China	Fleet	Australia
X151	Tibet, China	Gairdner	Australia
X026	Tibet, China	Numar	USA
X113	Tibet, China	Yerong	Australia
X076	Tibet, China	ZUG293	Sudan
X133	Tibet, China		
X061	Tibet, China		
X120	Tibet, China		
X097	Tibet, China		
X040	Tibet, China		
X161	Tibet, China		
X123	Tibet, China		
XZ115	Tibet, China		
X118	Tibet, China		
X051	Tibet, China		

Table 2.2 Selected wheat varieties and their origin

Bread wheat	Origin	Bread wheat	Origin
Albidum24	Russia	Xinong2000	China
Emai 19	China	Xinong223	China
Huanong5	China	Xiangmai25	China
Huaimai16	China	Yumai57	China
Kord Cl Plus	Australia	Zhoumai16	China
Liangxing99	China	Zhengmai9023	China
Linyuan8	China		
Ningmai17	China		
Onohoiskaja 4	Russia		
Pobeda	Russia		
Surhak Mestnyj	Tajikistan		
Tainong292	China		
Durum wheat	Origin		
Mahon demias	Spain		
Preto Amarelo	Portugal		

2.1.2 Experimental design and growth conditions

Three different types of glasshouse experiments were conducted in this study. The first experiment was performed to screen large barley and wheat germplasm for their drought tolerance using by complete withdrawal of irrigation and visual scoring of plant performance at three different time points. In the second experiment, the screening of all genotypes was conducted using three different water regimes (100%, 25% and 12% of field capacity) followed by physiological and agronomical assessment of plants. In the third experiment, genotypes contrasting in their tolerance to drought were selected from the previous experiments and whole plant traits were compared in control and water deficit irrigation.

Glasshouse screening based on a visual scoring

The experiment was set up in a randomized complete block design in the glasshouse of the Horticultural Research Center, University of Tasmania, Hobart in July-October 2015. Settings for the glasshouse were: 25/18 (±1) ^{0}C day/night temperature and 65/80 (±5) % day/night relative humidity. Large plastic tanks (60 cm length, 40 cm width; 62 L volume) filled with standard potting mixture were used. The composition of the potting mixture (by volume) was: 90% composted pine bark, 5% sand and 5% coir peat, plus additives gypsum (1 kg m^{-3}), ferrous sulphate (1.5 kg m^{-3}), magnesium sulphate (0.02 kg m^{-3}), copper sulphate (0.03 kg m^{-3}), osmoform (1.25 kg m^{-3}), osmocote (3 kg m^{-3}), and pH 6.0. At the bottom of each plastic container ten drilled holes were made in order to allow complete drainage of the container. This potting mixture was kept drained for one day before sowing of seeds. Two treatments, control and withhold irrigation, were applied (see details below 2.1.3). Eight tanks were used for each treatment. Both the control and stressed treatment area were divided into four blocks (randomized complete block design).

Screening based on physiological and agronomical traits

The study was conducted in the glasshouse of Tasmanian Institute of Agriculture (TIA), Hobart, Tasmania, in March 2016. The experiment was carried out using standard potting mixture and in randomized complete block design with three replications. The potting mixture was kept drained for 24 hours before sowing seeds. Before sowing seeds, the soil water holding capacity was measured (see details). Seeds were sown at a 10mm-depth in 2L PVC pots containing potting mixture for screening based on agronomical and physiological traits. Eight seeds per pot were sown. Plants were irrigated with a tap water and grown under controlled glasshouse conditions (day length, 14h; day/night temperatures, 25/15°C; relative humidity, 65%).

Comparing genotypes on the basis of whole plant traits

Seven barley varieties (*Hordeum vulgare* L.) were chosen from previous experiments based on their tolerance to drought (Table 2.3). The experiment was carried out in the glasshouse of Tasmanian Institute of Agriculture (TIA), Hobart, Tasmania from October 2016 to January 2017. The layout and growth conditions were the same of the following experiment except the soil. The sandy loam soil mixed with standard

fertilizers N (Urea 100kg/ha), P (Super phosphate 150kg/ha) and K (Muriate of Potash 50 kg/ha) was used.

Table 2.3 Selected barley varieties and their tolerance to drought stress

Variety	Tolerance to drought
Numar	Highly tolerant
ZUG293	Highly tolerant
Commander	Moderately tolerant
Fleet	Moderately tolerant
X123	Moderately tolerant
Gairdner	Highly sensitive
Franklin	Highly Sensitive

2.1.3 Experimental protocol

Screening based on a visual scoring

Seeds were surface-sterilized with 10% commercial bleach (sodium hypochlorite) (White King, Victoria, Australia) and thoroughly rinsed with tap water. Twenty-five genotypes were grown in one tank with five plants per genotype. Planting density was maintained as 5 plants per 9.6 cm^2. Watering was done twice daily until three to four leaves stage, and weeds were manually removed when observed. At three to four leaves stage irrigation was stopped for the stressed plants while normal irrigation was given for the control plants. Once the irrigation was stopped, the data was recorded for stressed treatment 3^{rd}, 5^{th} and 7^{th} week of withholding the irrigation. All plants grown under control conditions looked healthy and showed no symptoms of leaf chlorosis or dead leaves.

At each time point, the following information was collected:

- The total number of leaves per plant
- The number of chlorotic leaves
- The number of necrotic leaves

Based on above characteristics, a visual score (1-10) was allocated to each genotype, as a proxy of the drought-induced damage index (0 = no visual symptoms of stress; 10 = all plants are dead) as per table below (Table 2.4, Fig 2.1)

Table 2.4 Visual scoring of the drought-induced damage index

Drought score	Description
1	A green and healthy plant with no symptoms of stress effects
2	Bottom leaves beginning to yellow
3	Necrosis on a quarter (25%) of all leaves (normally the older leaves)
4	Necrosis on bottom half (50%) of all leaves
5	Necrosis on bottom half and yellowing to the upper half of the plant
6	Necrosis on more than the bottom half of the plant
7	Necrosis on 75% of the leaves
8	Necrosis on whole plant with apical leaves still green/chlorotic
9	Only stem and the shoot tips are green
10	Plant apparently dead (stem and leaves)

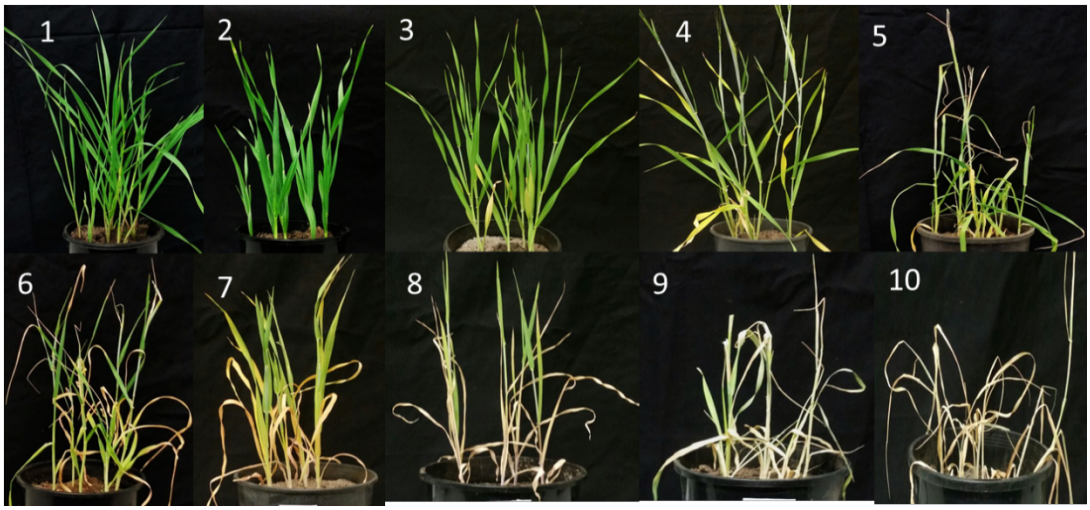

Figure 2.1 Visual scoring index (1-10) recorded by the end of seventh week after withholding the irrigation. 1= a green and healthy plant; 10= dead plant.

Screening based on physiological and agronomical traits

At two to three leaves stage, seedlings were subjected to three irrigation regimes, control (100% field capacity) and for stress treatments, soil water status was gradually brought down to specific field water holding capacity (FC = 25% and FC =12%) by weighing the pots daily at a fixed time of the day.

Determining the soil water holding capacity

Six uniform sized pots were filled with the soil and then watered to excess for drainage. Pots were allowed to drain overnight to obtain pot's wet weight (W_1). The pots were then allowed to dry in an oven at 60 ^oC until they reached a constant weight and dry weight (W_2) was recorded. Soil water content was determined by subtracting the dry weight from the wet weight ($Ws = W_1 - W_2$). Dry soil weight was determined by deducting the weight of the empty pot (W_P) from the pot dry weight ($W_D=W_2-W_P$). The target soil water content (W_T) was determined from the relative soil water content (% RSWC) by using equation:

$$W_T = W_P + W_D + \% \text{ RSWC} \times W_S$$

After six weeks of drought imposed, control and stressed plants were assessed for following measurements. The third youngest leaf was selected from control and stressed plants to record all measurements. Mark the leaves on the individuals (with cable ties) to ensure that the repeated measurements are done on the same leaf. Plant physiological (stomatal conductance; chlorophyll content; chlorophyll fluorescence) and agronomical (relative water content; shoot fresh weight; shoot dry weight) were then measured.

Comparing genotypes on the basis of the whole plant traits

At two to three leaf stage, seedlings were subjected to three irrigation regimes, control (100% field capacity) and two water deficit regimes (25% and 12% field capacity). After six weeks of drought imposed, control and stressed plants were assessed for following measurements:

- Root length
- Stomatal conductance
- Stomatal density
- Leaf water potential
- Relative water content
- Leaf and root K^+, Na^+ and Cl^-
- Leaf total soluble sugars
- Leaf total amino acids
- Leaf and root osmolality

2.1.4 Measurements

2.1.4.1 Assessment of physiological traits

Stomatal conductance

Stomatal conductance was measured using a Decagon Leaf porometer (Decagon devices, Inc. Pullman, WA, USA). Data was recorded under natural light conditions on a sunny day, between 10 am and 12 am. Leaf was placed into the chamber at the mid-point of the leaf in such a way that the selected area of the leaf completely covers the aperture of the sensor. Measurements were conducted from 12 plants of each cultivar exposed to drought and control conditions. Twelve readings were averaged to get mean value.

Leaf chlorophyll content

Leaf chlorophyll content was measured from the middle of the lamina of the third youngest leaf (selected for all measurements) using a SPAD chlorophyll meter (SPAD-502, MINOLTA, Japan). Twelve measurements were taken for each treatment.

Chlorophyll fluorescence

Photochemical efficiency of Photosystem II (chlorophyll fluorescence *Fv/Fm* ratio) was measured using a portable OS-30p chlorophyll fluorometer (Opti-sciences, Hudson, NH, USA). Plants were kept in the dark for 30 min prior to measurements. *Fv/Fm* values were measured from the middle of the third youngest leaf. Twelve replicates of each genotype/treatment were used.

Relative water content

The leaf relative water content (RWC), which is used as the reference value of water content, was determined by following equation (Zhang et al., 2012):

$$RWC = FW – DW/ FW \times 100$$

where FW is the fresh weight, and DW is the dry weight.

Leaf water potential

Leaf water potential was measured by the Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). All the collected leaves samples were wrapped in a plastic bag to prevent transpiration. Leaf blade was cut at the base using a razor blade. Place the leaf in the chamber with the cut end of the petiole protruding through the seal. Properly seal the leaf in the pressure chamber using the appropriate gasket. Pressure was applied until water appeared at the cut end of the petiole. This pressure equals the opposite of the leaf water potential.

Leaf and root Na^+ and K^+ content

The youngest fully expanded leaves were harvested from each pot (four replicates for each cultivar for both drought-stressed and control plants) 8 weeks after sowing. Roots were collected at the same time point. Leaves and roots samples were quickly frozen in eppendorf tubes. For measuring ion content, thawed samples were hand squeezed to extract all the sap as described elsewhere (Cuin et al., 2009). The sap was collected immediately with a micropipette and placed in another eppendorf tube to be placed in freezer. The sap was centrifuged at 8000 rpm for 10 minutes to remove debris. The sap was diluted by taking 50µl of the collected supernatant mixed with 10ml of distilled water. Na^+ and K^+ concentration was measured using flame photometer (Corning 410C, Essex, UK). Four replicates for each cultivar for both drought stressed, and control plants were assessed.

Leaf and root Cl^- content

The diluted sap solution (as above) was used for measuring chloride concentration using the non-invasive microelectrode MIFE system (UTas Innovation Ltd, Hobart, Australia). Microelectrodes were pulled from non-filamentous borosilicate glass capillaries (GC 150-10, Harvard apparatus Ltd, Kent, UK) using a vertical puller (PP-830, Narishige, Tokyo, Japan) followed by drying overnight in an oven at 225°C and salinized with tributylchlorosilane (90796, Fluka Chemicals). For further use, electrode tips were broken to achieve external tip diameters of 2–3 μm by moving electrode blanks against a flat glass surface using a micromanipulator. The dried and cooled microelectrodes were then back filled with 0.5 M KCl followed by front filling with chloride inophore (99408-0.1ML-F). The prepared Cl^- electrode was calibrated with KCl solutions of concentrations 250μM, 500μM and 1000μM. Electrodes with a Nernst slope of less than 50 mV per decade and/or correlation less than 0.999 were discarded from measurements. A standard non-polarising Ag/AgCl reference electrode was prepared by inserting a silver wire into a capillary containing 2% agar prepared in 1 M KCl. The tips of the microelectrodes were aligned and positioned in a small chamber containing 5ml of leaf /root sap solution. The reference electrode was also placed into this chamber. The data was recorded using MIFE CHART software (Shabala et al., 1997) for at least five minutes and Cl^- concentration was determined by taking the mean value of each measurement.

Osmolality

Leaf and root samples from control and drought stressed plants were harvested. Roots were carefully rinsed under running water to wash all the soil. The samples were placed in eppendorf tubes separately and froze overnight. The frozen leaf and root samples were thawed and squeezed in the eppendorf to extract the sap. The extracted sap was centrifuged for 15 minutes at 3600rpm. Approximately 20μl of the collected supernatant was measured for its osmolality using a vapour pressure osmometer (Vapro, Wescor Inc. Logan, UT, USA).

Total soluble sugars

500 mg of fresh plant material was extracted with 5 ml 80% ethanol in a centrifugation tube. The supernatant was removed after centrifugation at 4000 rpm for 5 minutes. The tubes containing supernatant were placed in water bath at 60 ºC for 30 minutes. This ethanol solution contained the soluble sugars. After the ethanol evaporated, 5ml of distilled water was added to the sugars left on the walls and at the bottom of the tubes. Total sugars were measured by anthrone sulphuric acid (Dubois et al., 1951). 0.2 g of anthrone was added in 8.3ml ethanol and 20ml deionized water. 100ml of 98% Sulphuric acid was carefully added under fume cover hood while the solution was constantly cooled on ice. The solution was mixed well until the anthrone was dissolved. Reagents must be yellow coloured. This reagent should be always freshly prepared. For the standard, 10 mg d-glucose dissolved in 100 ml deionized water was used, to cover the range up to 0.1 mg/ml.

During measurements, the test tubes were cooled on ice, so the condensate does not disintegrate. 50µl extract was added to a test tube. 1950µl anthrone reagent was added to the sample or standard. The solution was mixed well and put the test tubes in boiling water for 7.5 minutes to determine total soluble sugars and then rapidly cooled on ice**.** The test tubes were allowed to warm up at room temperature. When cooled, the absorbance of the developed blue green color was determined by a spectrophotometer (Spectronic 200) at 620 nm against a blank containing only water and anthrone reagent. A calibration curve using pure glucose was made and the data expressed as mg glucose / g DW.

Total free amino acids

All leaf samples were kept on ice to prevent protein degradation**.** 500mg of frozen leaf tissue was powdered in a pestle and mortar with liquid nitrogen. To this homogenate 5ml of 80% ethanol was added. The homogenate was transferred to 2ml microcentrifuge tube and was centrifuged for 10 minutes at 8000 rpm at 4ºC. The extraction was repeated twice, pooling then the residue and all the supernatants. The supernatant was transferred to fresh tube and used for the quantitative estimation of total free amino acids. For the standard, 50mg leucine was dissolved in 50mL of distilled water in a volumetric flask. 10mL of this stock standard was taken and diluted to 100mL in another flask for working standard solution. A series of volume from 0.1 to 1mL of this standard solution gives a concentration range 10mg to 100mg proceeded

as that of the sample, and absorbance of the light was measured. A ninhydrin solution was prepared by dissolving 0.8g stannous chloride ($SnCl_2.2H_2O$) in 500mL of 0.2M citrate buffer (pH 5.0). This solution was added to 20g of ninhydrin in 500mL of methyl cellosolve (2 methoxyethanol). During measurements, 0.1ml of plant extract was taken and 1ml of ninhydrin solution was added. Volume was made to 2ml with distilled water. The tube was heated in boiling bath for 20minutes. 5ml of diluents (equal volumes of water and *n*-propanol) was added, and the contents mixed. After 15 minutes of developing the reaction, the absorbance of the light against a reagent blank was read in a spectrophotometer (Spectronic 200) at 570nm. A calibration curve was made using absorbance versus concentration.

2.1.4.2 Assessment of agronomical and anatomical traits

A collective sample of the shoot biomass for all the plants in each pot (five plants in total) was taken. The shoot was cut 1cm above the soil and its fresh weight was recorded immediately by using analytical balance. After recording fresh weight, the samples were then dried for 72 hours at 65°C in the drying oven and weighed using a digital balance. Roots were thoroughly washed with tap water, and the total root length of primary roots was measured.

Stomatal density in barley leaves was quantified by making leaf imprints. The abaxial leaf surface of the middle part of the third youngest leaf was coated with thin layer of clear nail varnish. The dried layer of the nail varnish was then peeled off using tweezers and placed on a glass slide in a manner that imprinted surface should be on upper side and covered with a coverslip. The imprints were examined at 100 × magnification (Leica DM500, Leica Microsystems). The number of stomata was then counted for each field of view and the stomatal density (number of stomata per unit of surface area) was calculated. For each of the genotype/treatment, three imprints from four biological replicates were analyzed.

2.1.5 Data Analysis

The data collected from all measured parameters were analysed by IBM SPSS statistics 20 (IBM, New York, USA). All results are given as means ± s.e. Bivariate correlation based on two-tailed Pearson's Correlation was used to determine the significant correlation between the characteristics that has been measured. Different low-case letters in each panel of the figures indicate significance at $P < 0.05$. Genotypes based on stomatal conductance, chlorophyll content, chlorophyll fluorescence, relative water content, shoot fresh and dry weight were grouped using Hierarchical cluster analysis (HCA) based on Euclidean distances as a measure of dissimilarity and Ward's method as a clustering algorithm using XLSTAT software (Addinsoft, New York, NY, USA).

2.2 Electrophysiological Experiments

2.2.1 Plant material and growth conditions

Seven barley varieties (*Hordeum vulgare* L.) contrasting in their tolerance to drought were chosen from previous whole plant study experiments (Table 2.3). The seeds were obtained from the Australian Winter Cereal Collection and multiplied in Launceston using TIA facilities. All seeds were surface sterilized with 10% commercial bleach available (sodium hypochlorite) (White King, Victoria, Australia) for ten minutes followed by a thorough rinsing under the tap water for 30 minutes to ensure the absence of residual bleach on the seeds. Once the rinsing was completed, seeds were placed in cavities of a punched plate sited on the top of a 500 ml container containing Basic Salt Medium (BSM) (0.5mM $CaCl_2$ and 1mM KCl and 1mM NaCl). Plants were grown hydroponically in the tanks for 4 days in dark with 24°C/ 20°C day/night temperature. The solution inside each container was aerated by an air stone connected to an air pump.

2.2.2 Ion selective flux measurements

Net fluxes of K^+, Na^+ and Cl^- were measured non-invasively using ion-selective vibrating microelectrodes (the MIFE^{TM} technique; University of Tasmania, Hobart,

Australia) previously described (Newman, 2001; Shabala and Shabala, 2002). MIFE microelectrodes were prepared on a daily basis. This process included several steps.

I. ***Preparing electrodes blanks:*** Electrodes were pulled out from non-filamentous glass capillaries (Harvard Apparatus, 30-0053, GC150-10) to tip diameter ~1 μm. The pulled electrodes were dried in the oven at 225°C overnight, and salinized with tributylchlorosilane (90796, Fluka Chemicals) to make their surface hydrophobic. The prepared electrodes were then able to be stored for several weeks under the cover.

II. ***Filling up electrodes:*** On the day of the measurement, an electrode blank was mounted on a microscope stage of a filling station and the electrode tip was broken against a flat glass surface to 2-3 μm in diameter. The electrode then was backfilled with the appropriate bckfilling solution followed by a front filling with the respective liquid ion exchanger (LIX) (Table 2.5). Special attention was paid to the absence of air bubbles in the electrode tip and the length of LIX shaft that should not exceed 200 μm in length. Prepared electrodes were placed in an electrode holder filled with BSM and left for conditioning. The Cl^- selective electrodes require up to half an hour for conditioning. Other ion selective electrodes used (K^+ and Na^+) are ready for use immediately after preparation.

III. ***Calibrating ion selective microelectrodes:*** Prepared ion selective electrodes were calibrated in a set of three respective standards that cover the range of the expected ion concentration (Table 2.6). The quality of prepared electrodes was assessed after the calibration. The electrodes with a slope below 50 mV/decade for monovalent ions and correlation below 0.999 were discarded from use (Shabala and Shabala, 2002).

Table 2.5 Specific details about the major types of commercially available LIX and backfilling solution

Ion	Catalogue No	Ionophore	Backfilling Solution (mM)
K^+	60031	Valinomycin	200 KCl
Na^+	71176	N,N',N''-Triheptyl-N,N',N''-trimethyl-4,4',4''-propylidynetris (3-oxabutyramide)	500 NaCl
Cl^-	24902	5,10,15,20, Tetraphenyl-21H,23H-porphin manganese (III) chloride	200 KCl

Table 2.6 Calibration standards of electrodes

Measured Ion	1st Calibration Standard	2nd Calibration Standard	3rd Calibration Standard
K^+	250µM	500µM	1000µM
Na^+	500µM	1000µM	2000µM
Cl^-	1000µM	2000µM	4000µM

Prepared microelectrodes were placed in electrode holders over a microscope stage, centered with ~3 µm spacing between them and co-focused. Two ions were measured simultaneously and essentially from the same site on the root. A measuring chamber with the immobilized root was placed on a microscope stage and the microelectrodes were positioned ~40 µm away (position M1) from the respective zone and the measurements were resumed. Electrodes were moved ~120 µm away from the root (to position M2) and back to position M1 with 6 sec recordings at each position. The fluxes for K^+, Na^+, and Cl^- were measured. Six types of different experiments were performed using the MIFE technique.

2.2.3 Experimental protocols

Profiling steady net K^+, Na^+ and Cl^- fluxes along the root

Three days old seedlings grown in BSM medium were treated with 200mM mannitol for 24 hour by replacing BSM with BSM containing 200mM mannitol. The roots were gently secured horizontally in a measuring chamber with a Parafilm strip and small plastic blocks. The Petri dish containing 4 days old barley seedlings was filled with 200mM mannitol solution made in BSM and seedlings were conditioned for 30 mints. Roots were measured at 12 positions along the root axis to cover all functional root zones in the range from 0 to 50 mm from the root tip for approximately 2-3 minutes at each site, to enable steady readings. The chamber was then replaced with a new one, and the measurements resumed. Six to seven plants were assessed for each treatment.

Dose-dependency of ion flux responses to osmotic stress

In these experiment, transient net fluxes of K^+, Na^+ and Cl^- were measured from two functionally different root zones (elongation and mature) of two contrasting genotypes in response to increasing concentration of mannitol. Four days old seedlings grown in BSM solution were used. Ion fluxes were measured for the first five minutes in control (bath solution), and then the mannitol treatment (20 to 400mM range) was given, adding the appropriate amount of the stock solution to the bath. The solution was quickly and thoroughly mixed by sucking and expelling using a Pasteur pippete, and the fluxes were measured for 15 min after applying each concentration. The time required for stock addition, mixing and establishing the diffusion gradients was about 2min (discarded from analysis).

Comparing transient ion responses of seven genotypes

The most appropriate concentration (200mM mannitol) was selected from the following experiment. Four days old seedlings of seven genotypes contrasting in drought tolerance were grown in BSM. Ion fluxes (K^+, Na^+, Cl^-) of root mature (20mm from tip) zone were measured for the first five minutes for control. Then the stress (200mM mannitol) was added to the petri dish containing roots and fluxes were measured for next 30 min after applying stress.

Steady state measurements in mature zone

Seedlings of seven contrasting genotypes were grown in Basic Salt Medium (BSM) for three days and then replaced with BSM containing 200mM mannitol and kept them for 48 hours. Steady state fluxes for K^+, Na^+ and Cl^- were measured in mature zone (~ 20mm) for 3-5 mints.

Membrane potential measurements

Membrane potential (MP) was measured from epidermal cells of intact roots of barley genotypes. The roots were gently secured horizontally in a measuring chamber with a Parafilm strip and small plastic blocks. The measuring chamber was containing BSM (for control) and BSM solution containing 200mM mannitol (for hyperosmotic stress) and conditioned for at least 30 minutes. The MP measurements were performed as described previously (Cuin and Shabala, 2005; Gill, Muhammad Bilal et al., 2017). The borosilicate glass microelectrodes (Clarke Electrochemical Instruments, Reading, UK) were filled with 1M KCl, connected to an IE-251 electrometer (Warner Instruments, Hampden, CT, USA) via an Ag–AgCl half-cell. For MP measurement, the microelectrode with a tip diameter of 0.5 µm was impaled into the epidermal cells of the mature root zone with a manually operated 3D micromanipulator (MHW-4, Narishige, Tokyo, Japan). MP values were recorded by the MIFE CHART software for at least 2 min after stabilization. MP values were measured from roots of 5–6 individual seedlings. At least four different cells were measured for each seedling.

Transient ion flux measurements in response to ABA

Four days old barley seedlings were grown in BSM. Net ion fluxes (K^+, Na^+, Cl^-) were measured for five minutes, and then ABA was added to the bath to achieve the working concentration of 10µM. Transient net ion fluxes were measured for further 30 minutes.

2.2.4 Data analysis

Ion fluxes were calculated using the MIFEFLUX software assuming cylindrical geometry of the root, taking into account specific parameters of the object in study (root radius, and the initial distance between the root and the electrodes at M1 position). The calculated flux file was transferred to a personal computer and Excel was used for data analysis and graphing. The average data from 6-7 replicates were analyzed by IBM SPSS statistics 20 (IBM, New York, USA). Standard least significant difference

test at $P \leq 0.05$ was used to confirm the significant difference between treatments and varieties. Bivariate correlation was used to determine the significant correlation between the characteristics that had been measured.

Chapter 3. Assessing the extent of genetic variability in drought tolerance in wild and cultivated barley germplasm

3.1 Introduction

Drought is one of the major constraints limiting crop production globally. It has been estimated that climate change, along with warming temperatures, will exacerbates drought in the next 30–90 years that will affect over one-third of the earth as a result of both decreased precipitation, increased evaporation or both (Cook et al., 2014; Dai, 2013). This is expected to result in significant (over 75%) losses in agricultural production worldwide, costing approximately $23.5 billion per year and posing a major risk to food security (FAO, 2015). At the same time, global food demand is predicted to grow by 70–85% as the population increases to over 9 billion people by 2050 (FAO, 2017; Ray et al., 2013).

Barley (*Hordeum vulgare* L.) is the fourth largest cereal widely grown in the world. It is used as human feed, animal food and beverages (Nadira et al., 2014). However, as a result of a long process of domestication, including modern breeding and cultivation programs, primal landraces have been replaced by the modern cultivars. This genetic erosion in many domesticated plants has been under way for decades, In barley, many of the ancient landraces have vanished, and the genetic diversity of the cultivated forms has become significantly reduced (Ma et al., 2012; Zhao et al., 2010). Due to rapid loss of genetic variation, and as a result of selection for yield but not tolerance, modern barley cultivars have become sensitive to abiotic and biotic stresses (Ahmed et al., 2013). However, given remarkable genetic diversity, barley genotypes exhibit different responses to unfavorable environmental conditions, including drought stress (Marok et al., 2013). Which of them are most essential to confer the drought tolerance? No clear answer is available in the literature, prompting for a need to evaluate the genotypic variations in barley for drought tolerance and to detect some important agro-physiological traits for efficient and rapid screening in large germplasm. Also, wild barley relatives may have many desirable traits, including tolerance for drought stress, which could be used for barley improvement (Johnston et al., 2009). Which of them may be most suitable for breeders?

The degree of plant drought tolerance differs not only among various species but also among different varieties of the same species (Khalili et al., 2013). Species tolerant to drought generally differ morphologically and/or physiologically and possess mechanisms allowing better production under limited water supply (Pinheiro et al., 2005). Drought tolerance of a crop is usually related with its ability to maintain high yields under moisture deficit conditions (Dbira et al., 2018). As yields depends on growth and development of plants, when selecting drought tolerant genotypes, the relative reduction in growth under water deficit is believed to an optimal indicator of adaptive capacity (Chen et al., 2016). Unlike field trials, drought stress treatments imposed during vegetative growth stage under glasshouse conditions are less costly, easier and the intensity of the stress applied may be easily monitored; therefore, are ideal for selection of efficient markers of stress in plants (Chen et al., 2016; Schiop et al., 2015). At vegetative growth stage, fresh shoot weight and dry shoot weight are prime parameters to evaluate the inhibitory effects of drought among different genotypes (Guo et al., 2018).

To quantify the tolerance level, the most common and traditional method is a visual scoring of the extent of damage, based on number of necrotic and chlorotic leaves (on 0 to 10 scale) (Maliro et al., 2008; Swapna and Shylaraj, 2017). Although this approach is fast and straightforward, concerns have been raised which question the validity of this technique (Mantri et al., 2014). Visual scoring based on naked eye may not adequately explain the physiological status of plants (Masuka et al., 2017). Moreover, this method did not distinguish between tolerance and avoidance mechanisms (Ingram et al., 1990).

The screening done on the basis of yield and yield related traits is arguably the most accurate but also time consuming and labor intensive as it requires the screening trial to last through the entire plant ontogeny. Therefore, different physiological (stomatal conductance, chlorophyll content, Fv/Fm and relative water content) and agronomical (biomass) traits measured at early stages of plant development are often used as suitable proxies. Under drought conditions, the rapid response is the closure of stomata to avoid excessive water loss. However, the avoidance of excessive water occurs at the expense of reducing the CO_2 availability inside the leaf (Chaves et al., 2002). A high level of oxygen produces reactive oxygen species that causes rupturing of membranes, consequently affecting respiration, photosynthesis, and the overall development of the plant (Ahmad et al., 2018). Therefore, stomatal conductance is believed to be a suitable

proxy to measure plant transpiration and CO_2 assimilation in plants under water deficit conditions (Yan et al., 2016). As drought stress can accelerate chlorophyll decomposition, chlorophyll content is one of the most frequently used tools for evaluating the severity of drought stress. Water stress remarkably diminished chlorophyll content of plants (Gholamin and Khayatnezhad, 2011; Mafakheri et al., 2010) ultimately diminished plant growth and yield. The results, however, may be somewhat misleading, as although drought stress negatively affects chlorophyll biosynthesis, chlorophyll density per unit of area may increase as a result of reduced leaf growth and thicker leaves in stressed plants (Hasanuzzaman et al., 2017; Maréchaux et al., 2015). Chlorophyll fluorescence specifically, the maximum quantum efficiency of light harvesting in PSII (*Fv/Fm* ratio) in dark adapted leaves is another effective and reliable diagnostic tool for evaluating the plant germplasm for drought tolerance (Hasanuzzaman et al., 2017; Li et al., 2006). Other chlorophyll fluorescence characteristics such as PSII operating efficiency (*Fq/Fm*), PSII maximum efficiency (*Fv /Fm*) and the PSII efficiency factor (*Fq/Fv*) may also be used (Ghotbi-Ravandi et al., 2014; Oukarroum et al., 2009). Leaf relative water content (RWC) is also considered as an important indicator of water status in plants; it reflects the balance between water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca, 2011). The lowest water loss values by leaves are associated with a high drought tolerance (Ciulca et al., 2009; Gholami et al., 2012).

Though different strategies have been proposed for selection of genotypes, but it is still unclear that which technique is the most appropriate and what agronomical and physiological characteristics are most suitable to use as a proxy for drought tolerance. Moreover, to the best of our knowledge, no study has been conducted on barley in order to compare the results of visual scoring technique and physiological measurements. It also remains to be answered which specific mechanisms (of many involved; see Chapter 1) plays a major role in drought tolerance in barley.

The aim of the study was three-fold. First, we aimed to evaluate the extent of genotypic variations among barley genotypes in response to drought stress to reveal which cultivar better adapts to water stress conditions. The second objective was to compare various screening techniques and offer plant breeders a convenient method for the rapid assessing stress tolerance in barley germplasm. Last but not least, by comparing wild and cultivated barley genotypes we aimed at understanding the physiological basis of the drought tolerance traits in barley.

3.2 Results

3.2.1 Glasshouse screening based on visual scoring

The first glasshouse experiment for screening drought tolerance in barley was carried out in large tanks filled with potting mixture (see 2.1.2 for details). The irrigation was withheld when plants were 14 days old, and then exposed to progressive drought for 49 days. Due to the large tank volumes, plants retained moisture for longer time, therefore we recorded data at three different time points i.e., 3rd, 5th and 7th week after irrigation was withheld. Drought tolerance of wild and cultivated barley germplasm was evaluated on the basis of total number of leaves, number of chlorotic and necrotic leaves and visual scoring. Variety Flagship exhibited the highest number of leaves per plant i.e., 17.75 ± 0.25, while genotype X040 showed the least number of leaves per plant (8.75 ± 0.25) at the end of seventh week of drought stress (Table 3.1). In case of number of chlorotic leaves, X061, ZUG293 and X115 produced highest number of chlorotic leaves (3.00 ± 0.00, 1.75 ± 0.48, 2.00 ± 0.41 respectively) at third, fifth and seventh week. However, genotypes X051 and Franklin developed no chlorotic leaves at the end of seventh week (Table 3.2) and thus deemed as drought tolerant.

Table 3.1 Total number of leaves including chlorotic and necrotic leaves at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
X112	14.25±0.48	14.25±0.48	13.75±0.48
X113	14.00±0.41	14.00±0.41	14.00±0.41
X115	14.00±0.71	14.00±0.71	13.75±0.71
X117	13.00±1.08	13.00±1.08	13.00±1.08
X118	13.50±0.29	13.50±0.29	13.50±0.29
X120	14.50±0.29	14.50±0.29	13.25±0.29
X123	14.25±0.63	14.25±0.63	14.25±0.63
X133	14.75±0.25	14.75±0.25	14.75±0.25
X151	11.00± 0.71	11.00±0.71	10.75±0.71
X161	9.50±0.50	9.50±0.50	9.50±0.50
X165	11.50±0.50	11.50±0.50	11.50±0.50
X026	9.75±0.48	9.75±0.48	9.75±0.48
X030	13.25±1.11	13.25±1.11	13.25±1.11
X040	8.75±0.25	8.75±0.25	8.75±0.25
X045	9.00±0.41	9.00±0.41	9.00±0.41
X051	9.00±0.41	9.00±0.41	9.00±0.41
X061	11.75±0.95	11.75±0.95	11.75±0.95
X076	12.00±0.58	12.00±0.58	12.00±0.58
X097	11.75±0.25	11.75±0.25	10.50±0.25
XZ115	13.75±0.75	13.75±0.75	13.75±0.75
Franklin	11.75±0.63	11.75±0.63	11.75±0.63
Gairdner	11.75±0.63	11.75±0.63	11.75±0.63
Commander	12.25±0.63	12.25±0.63	12.25±0.63
Fleet	13.25±0.75	13.25±0.75	13.25±0.75
Clipper	14.00±0.41	14.00±0.41	14.00±0.41
ZUG293	11.00±0.71	11.00±0.71	11.00±0.71
Yerong	12.50±0.87	12.50±0.87	12.50±0.87
CM72	14.75±0.25	14.75±0.25	14.75±0.25
Numar	16.25±0.48	16.25±0.48	16.25±0.48
Flagship	17.75±0.25	17.75±0.25	17.75±0.25

Table 3.2 Total number of chlorotic leaves at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
X112	1.50±0.29	0.75±0.25	0.75±0.25
X113	1.50±0.29	1.50±0.29	2.25±0.48
X115	1.75±0.25	1.50±0.29	2.00±0.41
X117	1.50±0.29	1.00±0.41	1.25±0.25
X118	1.25±0.25	0.50±0.29	1.00±0.41
X120	1.50±0.29	0.50±0.29	1.25±0.25
X123	2.25±0.25	0.25±0.25	1.00±0.41
X133	2.25±0.25	1.00±0.41	1.25±0.25
X151	1.50±0.29	0.75±0.25	0.75±0.25
X161	2.00±0.41	0.50±0.29	1.00±0.00
X165	2.00±0.41	0.50±0.29	0.50±0.29
X026	1.75±0.25	0.75±0.25	0.50±0.29
X030	1.50±0.29	0.25±0.25	0.50±0.50
X040	2.00±0.41	0.75±0.48	0.75±0.48
X045	2.25±0.25	0.75±0.25	0.75±0.25
X051	2.25±0.48	0.00±0.00	0.00±0.00
X061	3.00±0.00	0.25±0.25	0.25±0.25
X076	2.00±0.00	0.50±0.29	0.50±0.29
X097	2.00±0.41	0.50±0.29	0.75±0.25
XZ115	2.25±0.25	0.25±0.25	0.25±0.25
Franklin	1.75±0.25	1.00±0.41	0.00±0.41
Gairdner	0.75±0.25	0.50±0.29	1.00±0.41
Commander	0.50±0.29	1.00±0.41	1.00±0.41
Fleet	0.50±0.29	0.50±0.29	1.25±0.25
Clipper	1.00±0.41	0.25±0.25	0.25±0.29
ZUG293	1.25±0.29	1.75±0.48	1.00±0.48
Yerong	0.50±0.29	0.75±0.25	0.75±0.25
CM72	0.50±0.29	0.50±0.29	0.50±0.29
Numar	1.00±0.41	1.00±0.41	0.75±0.25
Flagship	1.50±0.29	0.50±0.29	0.50±0.29

Drought tolerance was also assessed by the total number of necrotic leaves produced by drought (Table 3.3). After three weeks of withholding the irrigation, the number of necrotic leaves varied between 2.75 ± 0.25 (X026) and 0.50 ± 0.29 (Fleet). All the genotypes showed an increase in the number of necrotic leaves as the stress developed. At 5th week of drought, the number of necrotic leaves ranged between 11.00 ± 0.41 to 4.25 ± 0.48. The maximum number of necrotic leaves was exhibited by X120 and the minimum number of necrotic leaves was found in Numar. By the end of 7th week, the average number of necrotic leaves varied between 13.50 ± 0.63 and 6.00 ± 0.25. The maximum number of necrotic leaves were produced by Gairdner, and the minimum number of necrotic leaves was found in Numar followed by ZUG293.

Visual scoring was assigned to all the genotypes after 3rd, 5th and 7th week of drought imposed (Table 3.4). In the third week, the visual scoring varied between 3.00 ± 0.00 and 0.75 ± 0.25. The highest score was exhibited by genotype X051and the lowest by cultivar Fleet. At week 5 of the stress, the visual scoring ranged between 8.75 ± 0.25 and 5.50 ± 0.29, and at week 7 the highest damage score was 9.75 ± 0.85 (for X117) and the lowest was 5.50 ± 0.29 (for ZUG293) (Table 3.4).

Table 3.3 Total number of necrotic leaves at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
X112	1.75±0.25	8.25±0.25	12.25±0.85
X113	1.50±0.29	5.75±0.25	9.50±1.04
X115	1.75±0.25	8.50±0.65	11.00±0.71
X117	1.00±0.41	7.00±0.91	11.00±1.00
X118	1.50±0.29	7.50±0.65	9.50±0.65
X120	2.50±0.29	11.00±0.41	11.75±0.48
X123	2.50±0.29	7.75±0.75	10.50±1.32
X133	1.75±0.25	8.00±0.41	11.25±0.48
X151	2.00±0.41	5.75±0.63	9.25±0.85
X161	2.25±0.25	5.00±0.71	9.75±0.85
X165	2.50±0.29	5.50±0.29	9.50±0.65
X026	2.75±0.25	6.00±0.41	7.00±0.41
X030	2.50±0.29	8.50±0.87	11.00±1.35
X040	2.50±0.29	5.75±0.48	7.25±0.63
X045	2.50±0.29	4.75±0.25	6.75±0.48
X051	2.50±0.29	7.25±0.25	6.25±0.25
X061	2.50±0.29	7.75±0.75	9.00±0.71
X076	2.50±0.29	7.00±0.58	9.75±0.85
X097	2.50±0.29	5.50±0.29	9.00±0.41
XZ115	2.25±0.48	7.00±0.41	10.00±0.41
Franklin	1.00±0.41	7.75±0.63	9.50±0.65
Gairdner	1.00±0.41	7.75±0.48	13.50±0.63
Commander	0.75±0.25	7.00±0.41	9.50±1.19
Fleet	0.50±0.29	8.75±0.85	10.00±0.41
Clipper	1.00±0.41	10.50±0.50	12.50±0.50
ZUG293	1.25±0.48	5.75±0.48	6.75±0.48
Yerong	0.75±0.48	7.25±0.48	7.00±0.58
CM72	1.25±0.48	9.25±0.25	8.75±0.25
Numar	1.50±0.29	4.25±0.48	6.00±0.25
Flagship	1.00±0.41	9.75±0.25	9.50±0.29

Table 3.4 Visual scoring of the genotypes at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
X112	2.50±0.29	8.25±0.25	9.25±0.25
X113	2.00±0.58	8.50±0.29	9.00±0.41
X115	1.75±0.25	7.75±0.25	9.50±0.29
X117	1.75±0.25	7.25±0.25	9.75±0.25
X118	1.25±0.25	7.00±0.41	7.25±0.25
X120	2.25±0.25	8.50±0.29	9.50±0.29
X123	2.75±0.25	6.50±0.29	8.75±0.25
X133	2.50±0.29	6.75±0.25	8.50±0.29
X151	2.00±0.00	6.75±0.25	7.25±0.25
X161	2.50±0.50	7.25±0.25	9.00±0.41
X165	3.00±0.00	5.75±0.25	9.50±0.29
X026	2.75±0.25	5.50±0.25	6.50±0.29
X030	2.50±0.29	6.75±0.25	9.50±0.29
X040	2.25±0.48	8.75±0.25	9.75±0.25
X045	2.50±0.29	6.75±0.25	8.75±0.25
X051	2.75±0.25	8.00±0.00	7.00±0.00
X061	2.50±0.29	7.75±0.25	9.00±0.00
X076	3.00±0.00	7.25±0.25	9.25±0.25
X097	2.50±0.29	6.00±0.00	9.25±0.25
XZ115	2.50±0.50	6.00±0.00	6.75±0.25
Franklin	1.75±0.25	7.75±0.25	9.00±0.71
Gairdner	2.00±0.00	7.75±0.75	9.00±0.71
Commander	1.25±0.25	6.50±0.29	8.25±0.48
Fleet	0.75±0.25	7.25±0.25	8.50±0.65
Clipper	1.25±0.25	8.25±0.48	9.50±0.29
ZUG293	1.00±0.00	5.50±0.25	5.50±0.29
Yerong	1.00±0.00	6.75±0.25	6.75±0.25
CM72	2.00±0.00	7.00±0.00	6.75±0.25
Numar	1.75±0.25	5.75±0.48	6.50±0.29
Flagship	1.75±0.25	5.50±0.29	6.00±0.00

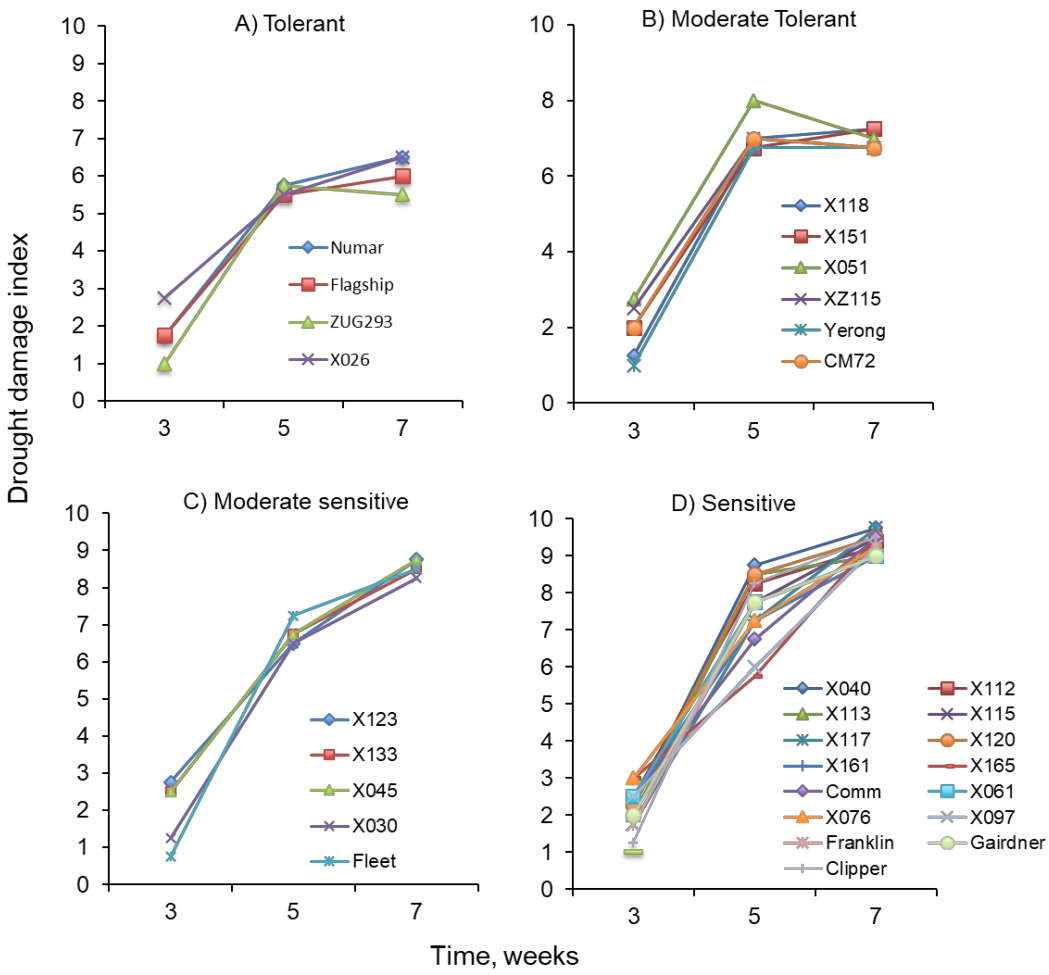

Figure 3.1 Four major groups were distinguished based on drought damage index: A, tolerant (DDI=5.5-6.5); B, moderately tolerant (DDI=6.75-7.25); C, moderately sensitive (DDI=7.5-8.75); D, sensitive (DDI=9-9.75)

Based on obtained visual scoring, all barley genotypes were clustered into four groups. The tolerant cluster contained varieties ZUG293, Flagship, Numar, and X026 (with the drought damage index 5.50-6.50). Moderately tolerant cluster included cultivars X118, X151, X051, XZ115, Yerong, CM72 (damage index between 6.75 and 7.25); moderately sensitive cluster contained genotypes X123, X123, X045, Commander, and Fleet (damage index between 7.25 and 8.75); and a sensitive cluster including genotypes X040, X113, X117, X161, X061, X097, Gairdner, X112, X115, X120, X165, X030, X076, Franklin, Clipper (damage index over 9) (Fig 3.1).

3.2.2 Analysis of physiological traits and biomass

The second glasshouse screening experiment was performed on the basis of physiological traits and biomass. The experiment was carried out in pots filled with a potting mixture under controlled irrigation conditions: full irrigation (control); and two water deficit irrigations (25% and 12% of full field capacity).

For all the traits the mean values were higher in control conditions as compared to drought stress. Under irrigated conditions, SPAD values differed significantly between genotypes, ranging between 39.5 ± 0.50 and 27.0 ± 0.92 (Table 3.5). The highest SPAD value was for genotype X113, and the lowest – for X123. Under 25% field capacity irrigation, chlorophyll content (SPAD) greatly reduced, ranging between 27.0 ± 0.73 and 3.1 ± 0.20. X133 had the maximum chlorophyll content whereas Gairdner had the lowest. More severe drought stress (12% of full field capacity) has further reduced SPAD values ranging between 18.2 ± 0.65 (X112) and 1.6 ± 0.11 (Franklin) (Table 3.6).

Table 3.5 Genotypic variability in chlorophyll content (SPAD) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	SPAD	25%FC	12%FC
X115	32.8±0.46	14.9±0.56	7.7±0.22
X117	28.9±0.12	14.5±0.72	5.4±0.26
X113	39.5±0.50	6.8±0.44	1.7±0.38
X120	33.5±0.32	14.4±0.53	3.7±0.19
X123	27.0±0.92	7.2±0.21	4.6±0.16
X030	32.1±0.25	13.1±0.51	9.5±0.22
X045	31.6±0.69	14.7±0.57	6.3±0.33
X076	35.9±0.64	22.2±0.55	16.9±0.54
X097	33.0±0.38	15.5±0.51	6.6±0.24
XZ115	31.5±0.39	14.5±0.44	4.2±0.21
X112	38.5±0.63	24.8±0.95	18.2±0.65
X151	35.3±0.54	25.3±0.93	13.2±1.14
X133	35.7±0.57	27.0±0.73	4.3±0.23
X040	33.2±0.48	19.4±1.89	5.1±0.38
X118	33.4±0.61	15.0±0.97	7.8±0.40
X165	32.1±0.74	17.2±0.64	4.1±0.54
X026	34.4±0.50	20.2±0.80	11.8±0.32
X061	34.7±0.48	25.9±0.52	15.4±0.77
X161	30.5±0.51	13.3±0.82	8.5±0.99
X051	38.5±0.60	13.3±0.82	5.1±0.32
Franklin	31.9±0.91	4.6±0.35	1.6±0.11
Gairdner	32.1±1.10	3.1±0.20	1.8±0.13
Commander	36.2±1.18	7.5±0.19	3.7±0.13
Fleet	32.5±0.54	4.0±0.28	8.3±0.27
Clipper	31.6±0.30	16.4±0.37	4.7±0.26
ZUG293	35.4±0.47	14.3±1.05	6.3±0.45
Yerong	28.5±0.28	14.6±0.53	10.2±0.32
CM72	35.1±0.43	13.9±0.52	2.7±0.25
Numar	29.6±0.25	19.1±0.82	16.8±0.44
Flagship	36.6±0.40	15.9±0.17	11.7±0.47

Under control irrigation, maximum quantum yield of PSII (*Fv/Fm*) was not significantly different between genotypes and close to 0.8, indicating fully functional PSII operation (Table 3.6). Chlorophyll fluorescence severely diminished among all the genotypes subjected to drought. *Fv/Fm* ratio varied between 0.726 ± 0.004 (Numar) and 0.419 ± 0.010 (X113) under 25% field capacity irrigation regime. Under severe drought stress conditions (12% of full field capacity), *Fv/Fm* values varied between 0.652 ± 0.009 (X061) and 0.268 ± 0.010 (Gairdner).

Table 3.6 Genotypic variability in chlorophyll fluorescence (*Fv/Fm*) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
X115	0.797±0.002	0.594±0.003	0.442±0.012
X117	0.786±0.003	0.550±0.008	0.509±0.010
X113	0.798±0.002	0.419±0.010	0.375±0.010
X120	0.780±0.004	0.582±0.009	0.365±0.011
X123	0.800±0.001	0.698±0.008	0.550±0.008
X030	0.791±0.003	0.695±0.006	0.644±0.008
X045	0.796±0.003	0.599±0.005	0.446±0.011
X076	0.786±0.004	0.641±0.008	0.589±0.008
X097	0.776±0.006	0.578±0.007	0.339±0.007
XZ115	0.780±0.004	0.554±0.007	0.300±0.012
X112	0.798±0.002	0.682±0.007	0.523±0.021
X151	0.800±0.002	0.711±0.005	0.599±0.002
X133	0.800±0.001	0.622±0.002	0.316±0.008
X040	0.794±0.002	0.622±0.002	0.512±0.013
X118	0.799±0.001	0.629±0.003	0.291±0.014
X165	0.799±0.002	0.732±0.005	0.431±0.020
X026	0.802±0.002	0.734±0.007	0.645±0.009
X061	0.800±0.001	0.717±0.004	0.652±0.009
X161	0.790±0.002	0.600±0.005	0.371±0.003
X051	0.787±0.002	0.526±0.005	0.323±0.005
Franklin	0.790±0.003	0.565±0.012	0.281±0.011
Gairdner	0.790±0.003	0.442±0.012	0.268±0.010
Commander	0.788±0.003	0.530±0.010	0.297±0.003
Fleet	0.796±0.002	0.697±0.005	0.549±0.008
Clipper	0.799±0.002	0.616±0.006	0.421±0.011
ZUG293	0.777±0.003	0.560±0.007	0.365±0.011
Yerong	0.786±0.003	0.532±0.005	0.320±0.004
CM72	0.756±0.006	0.514±0.010	0.337±0.011
Numar	0.800±0.001	0.726±0.004	0.633±0.010
Flagship	0.794±0.002	0.715±0.006	0.603±0.003

Under irrigated conditions, the highest stomatal conductance was found in cultivated Gairdner variety (57.4 ± 2.83) followed by Numar (54.2 ± 0.65). The lowest stomatal conductance was observed in wild barley X165 and X133 (30.7 ± 0.48) (Table 3.7). Under mild drought stress, stomatal conductance ranged between 29.7 ± 1.68 to 4.5 ± 0.69, with X026 having the highest Gs and X112 the lowest. Stomatal conductance was dramatically reduced under severe water stress, with Gs values ranging between 11.4 ± 0.49 and 0.4 ± 0.16. Genotype X030 had the highest stomatal conductance while Gairdner and X117 showed the least values of Gs (Table 3.7).

Table 3.7 Genotypic variability in stomatal conductance (Gs) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
X115	50.2±0.69	6.8±0.29	0.8±0.26
X117	41.9±0.62	7.3±0.33	0.4±0.19
X113	30.4±0.41	5.3±0.16	0.6±0.19
X120	41.6±0.39	9.1±0.19	2.3±0.34
X123	44.6±0.72	16.9±0.34	6.5±0.31
X030	55.7±1.35	28.9±0.92	11.4±0.49
X045	45.1±0.56	6.9±0.38	0.5±0.17
X076	35.2±0.77	16.5±0.43	7.6±0.25
X097	35.6±0.88	7.9±0.27	0.6±0.22
XZ115	41.5±0.57	8.9±0.25	0.9±0.27
X112	45.1±2.44	4.5±0.69	1.0±0.30
X151	48.6±1.23	7.6±0.69	1.3±0.36
X133	30.8±0.48	4.9±0.19	0.6±0.20
X040	45.1±2.44	9.8±0.87	0.9±0.24
X118	48.4±1.23	8.3±0.17	0.9±0.20
X165	30.7±0.48	17.8±0.26	6.1±0.70
X026	37.3±0.65	29.7±1.68	8.2±0.91
X061	41.4±0.28	10.7±1.10	8.3±0.46
X161	44.9±0.59	13.8±1.12	1.9±0.50
X051	52.3±1.86	14.4±1.08	2.8±0.72
Franklin	35.8±0.96	11.6±0.30	0.6±0.19
Gairdner	57.4±2.83	7.4±0.32	0.4±0.16
Commander	43.1±0.46	4.7±0.19	0.6±0.16
Fleet	33.8±0.55	7.4±0.18	0.6±0.20
Clipper	45.9±0.94	13.6±0.21	2.2±0.28
ZUG293	50.2±0.72	15.8±0.16	5.0±0.14
Yerong	43.5±0.62	11.6±0.22	0.6±0.22
CM72	39.4±1.01	9.5±0.42	0.7±0.23
Numar	54.1±0.65	16.3±0.21	10.5±0.68
Flagship	52.7±0.87	15.3±0.63	5.3±0.42

Fresh shoot weight ranged between 2.73 ± 0.42g (Flagship) and 0.71 ± 0.10g (Franklin) among the genotypes under control conditions (Table 3.8). However, under 25% field capacity irrigation, plant shoot fresh weight ranged between 1.42 ± 0.08 (X026) and 0.30 ± 0.05 (Gairdner). Shoot fresh weight varied between 0.90 ± 0.13g (X026) and 0.06 ± 0.04g (Gairdner) under severe drought stress. Genotype X026 had the highest shoot fresh weight and Gairdner had the least (Table 3.8).

Table 3.8 Genotypic variability in shoot fresh weight (FW) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
X115	1.27±0.39	0.51±0.07	0.35±0.02
X117	1.67±0.54	0.87±0.16	0.27±0.02
X113	1.70±0.05	1.10±0.06	0.21±0.01
X120	2.38±0.48	0.99±0.10	0.47±0.13
X123	1.23±0.14	0.65±0.07	0.39±0.06
X030	1.42±0.34	0.67±0.13	0.43±0.08
X045	1.41±0.46	0.72±0.22	0.32±0.07
X076	2.40±1.00	1.04±0.14	0.52±0.07
X097	2.20±0.32	0.58±0.14	0.40±0.00
XZ115	1.83±0.22	0.68±0.11	0.41±0.05
X112	1.66±0.05	0.52±0.04	0.36±0.04
X151	1.37±0.09	0.45±0.04	0.30±0.04
X133	1.24±0.10	0.52±0.06	0.24±0.03
X040	1.27±0.21	0.48±0.03	0.31±0.02
X118	1.44±0.04	0.48±0.08	0.27±0.04
X165	1.15±0.09	0.42±0.03	0.31±0.02
X026	1.80±0.22	1.42±0.08	0.90±0.13
X061	0.85±0.06	0.41±0.09	0.33±0.01
X161	1.09±0.13	0.48±0.05	0.29±0.03
X051	1.24±0.08	0.37±0.03	0.21±0.04
Franklin	0.71±0.10	0.35±0.04	0.10±0.00
Gairdner	1.40±0.34	0.30±0.05	0.06±0.04
Commander	1.16±0.20	0.63±0.02	0.45±0.10
Fleet	0.90±0.11	0.37±0.04	0.28±0.01
Clipper	1.24±0.25	0.60±0.05	0.39±0.03
ZUG293	1.06±0.24	0.79±0.11	0.59±0.02
Yerong	0.79±0.07	0.59±0.00	0.18±0.01
CM72	1.03±0.18	0.56±0.07	0.30±0.04
Numar	1.77±0.33	0.60±0.06	0.44±0.02
Flagship	2.73±0.42	1.33±0.17	0.60±0.15

The shoot dry weight also showed a broad range of variability among genotypes under control irrigation, ranging between 0.43 ± 0.13g and 0.13 ± 0.01g, with X076 having the highest and X165 the lowest SDW (Table 3.9). Under mild stress (25% of full field capacity), shoot DW ranged between 0.24 ± 0.01g (X117) and 0.08 ± 0.01g (Clipper). More severe drought stress (12% of full field capacity) has further reduced shoot dry weight. Shoot dry weight ranged between 0.17 ± 0.02g and 0.05 ± 0.02g. X076 produced highest biomass whereas Gairdner and Clipper produced lowest (Table 3.9).

Table 3.9 Genotypic variability in shoot dry weight (DW) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
X115	0.21±0.03	0.12±0.01	0.09±0.01
X117	0.31±0.01	0.24±0.01	0.11±0.01
X113	0.18±0.01	0.13±0.00	0.05±0.01
X120	0.24±0.04	0.15±0.01	0.09±0.01
X123	0.23±0.03	0.12±0.01	0.09±0.01
X030	0.29±0.06	0.11±0.01	0.08±0.01
X045	0.20±0.03	0.15±0.01	0.06±0.01
X076	0.43±0.13	0.23±0.03	0.17±0.02
X097	0.23±0.03	0.15±0.00	0.10±0.00
XZ115	0.24±0.01	0.16±0.02	0.10±0.00
X112	0.21±0.01	0.14±0.00	0.12±0.01
X151	0.17±0.01	0.14±0.01	0.10±0.00
X133	0.14±0.02	0.10±0.01	0.10±0.01
X040	0.16±0.02	0.14±0.02	0.11±0.01
X118	0.19±0.02	0.14±0.02	0.10±0.02
X165	0.13±0.01	0.09±0.01	0.08±0.00
X026	0.22±0.01	0.18±0.02	0.16±0.01
X061	0.34±0.23	0.12±0.04	0.09±0.00
X161	0.16±0.01	0.11±0.01	0.08±0.01
X051	0.15±0.00	0.11±0.00	0.09±0.01
Franklin	0.15±0.01	0.11±0.01	0.06±0.01
Gairdner	0.17±0.03	0.14±0.01	0.05±0.02
Commander	0.17±0.00	0.10±0.00	0.06±0.00
Fleet	0.17±0.01	0.09±0.01	0.07±0.01
Clipper	0.18±0.02	0.08±0.01	0.05±0.01
ZUG293	0.16±0.00	0.10±0.00	0.08±0.01
Yerong	0.18±0.02	0.11±0.01	0.06±0.01
CM72	0.15±0.02	0.11±0.01	0.07±0.01
Numar	0.22±0.04	0.17±0.04	0.14±0.00
Flagship	0.27±0.03	0.17±0.03	0.12±0.01

The relative water content in control plants was higher as compared to plants in drought stress and varied between 90.6% (Clipper) and 71.7% (X061) (Table 3.10). Relative water content adversely affected by drought stress in all barley genotypes. RWC varied between 88.5% (X113) and 52.2% (Gairdner) under 25% field capacity irrigation. However, plant RWC was ranged between 87.9% and 43.3% under 12% field capacity regime, with Clipper having the highest relative water content and Franklin the lowest (Table 3.10).

Table 3.10 Genotypic variability in relative water content (RWC) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
X115	83.7	76.5	73.6
X117	81.4	72.1	61.0
X113	89.4	88.5	51.0
X120	90.0	85.2	81.7
X123	81.8	81.0	77.0
X030	81.4	83.2	79.8
X045	86.0	79.9	80.2
X076	81.9	77.7	67.7
X097	89.4	74.6	75.0
XZ115	87.1	76.6	75.8
X112	87.5	72.6	67.0
X151	87.4	69.6	65.9
X133	88.4	81.2	79.2
X040	87.1	70.8	63.0
X118	87.0	71.5	63.7
X165	88.4	78.7	73.9
X026	87.6	87.1	82.6
X061	71.7	70.7	60.2
X161	85.3	76.4	72.1
X051	87.4	71.4	68.5
Franklin	78.4	69.5	43.3
Gairdner	87.4	52.2	48.9
Commander	85.6	84.2	80.8
Fleet	87.0	75.7	76.2
Clipper	90.5	86.7	87.9
ZUG293	86.5	87.3	85.3
Yerong	76.9	82.0	64.1
CM72	85.1	80.5	75.8
Numar	87.5	71.3	67.2
Flagship	90.2	87.0	80.0

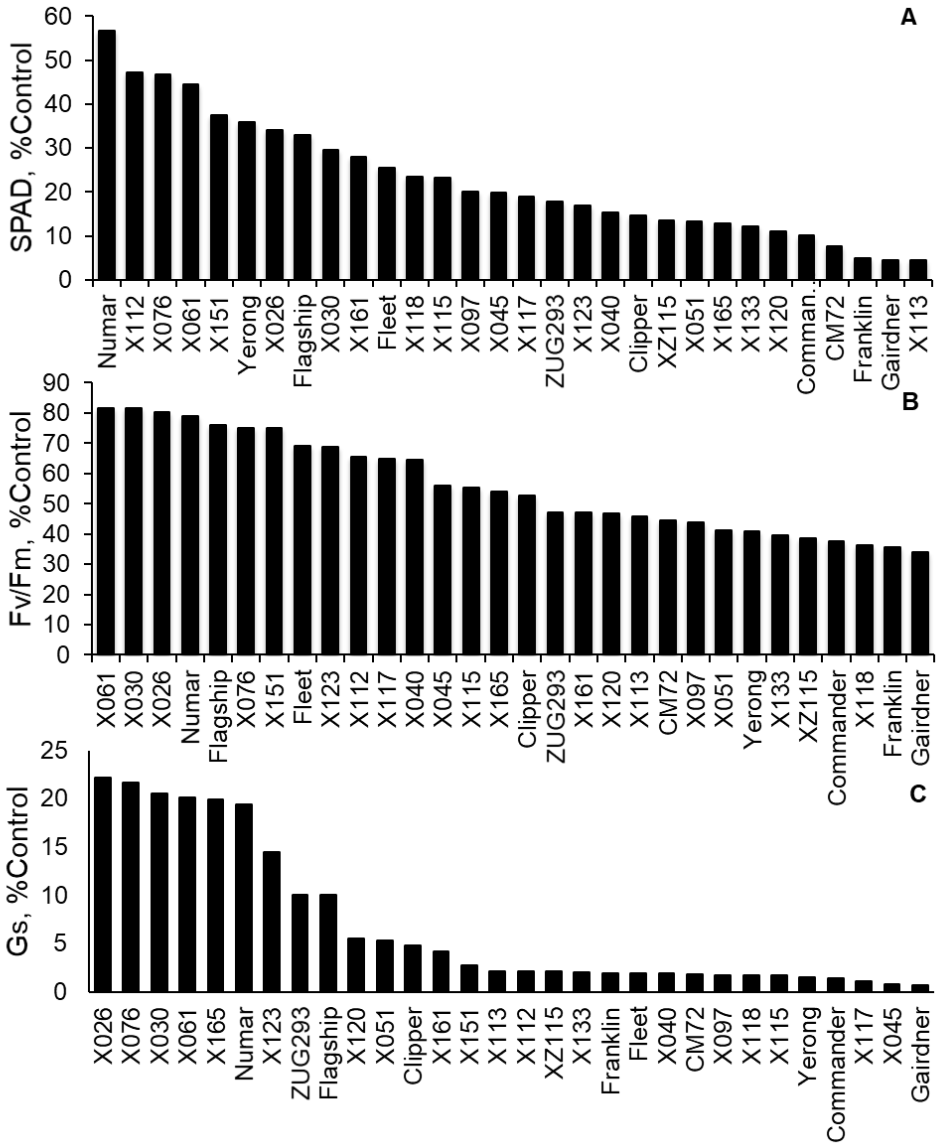

Figure 3.2 Relative chlorophyll content (SPAD) (A), chlorophyll fluorescence (*Fv/Fm*) (B), stomatal conductance (Gs) (C) of barley genotypes grown under severe drought stress (12% of full field capacity) shown as percentage of values under irrigated conditions

The relative changes in chlorophyll content (% control) differed among varieties (Fig. 3.2A) and ranged between 56.72% for Numar and as little as 4.44% for X113. The relative values of chlorophyll fluorescence of drought-stressed plants (% control) ranged between 81.58% for X061 and 33.86% for Gairdner (Fig 3.2B). On average, the stomatal conductance in drought stressed plants ranged between 22.7 % and 0.70% of the control, depending on the variety (Fig 3.2C).

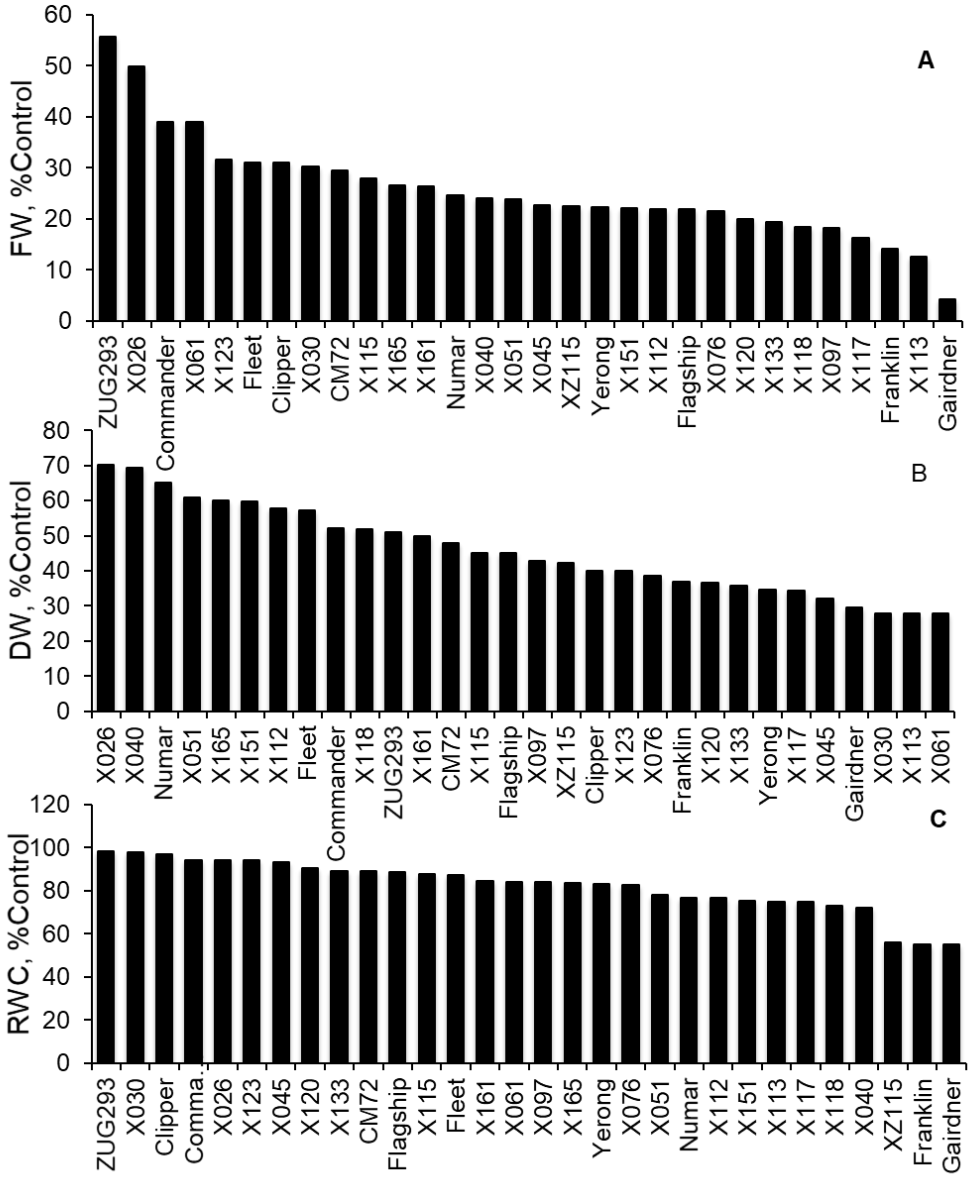

Figure 3.3 Relative fresh weight (FW)(A), dry weight (DW)(B), relative water content (RWC)(C) of barley genotypes grown under severe drought stress (12% of full field capacity) shown as percentage of values under irrigated conditions

The relative fresh weight values of drought stressed plants (% control) varied between 55.82% and as low as 4.82% with ZUG293 having the highest value and Gairdner the lowest one (Fig 3.3A). For shoot dry weight, the relative values of stressed plants ranged between 70.15% (X026) and 27.72% (X061) (Fig 3.3B). The relative water content in drought stressed plants ranged between 99% and 55%. The lowest reduction in RWC was exhibited in ZUG293 and the highest reduction was in Gairdner (Fig 3.3C).

3.2.3 Correlation analysis

No significant correlation was found between SPAD and shoot dry weight under irrigated conditions. A strong positive correlation (R^2=0.23; significant at *P<0.01* and R^2=0.47; significant at *P<0.001*) was seen between SPAD values of plants grown under moderate (25% field capacity) and severe (12% field capacity) stress and dry biomass at severe stress (Fig 3.4B&C).

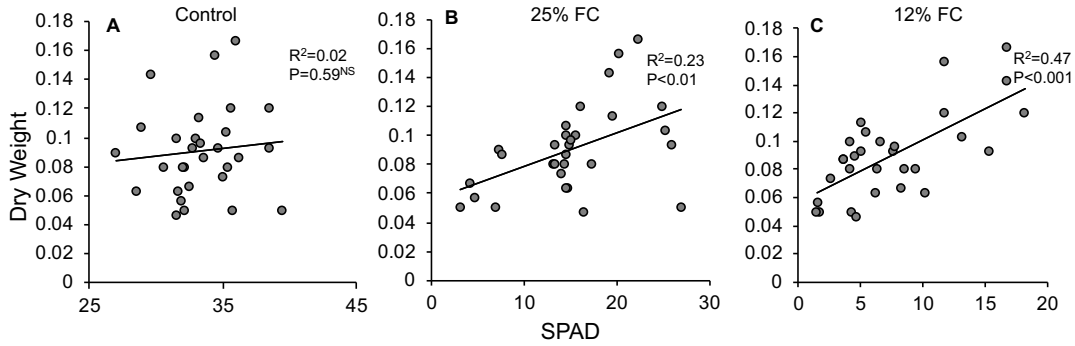

Figure 3.4 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and chlorophyll content (SPAD) of 30 barley genotypes grown under control, 25% and 12% field capacity conditions

A strong positive correlation was found between shoot dry weight and *Fv/Fm* of plants grown under moderate (25%FC) and severe (12% FC) stress conditions (R^2=0.25; significant at *P<0.01* and R^2=0.35; significant at *P<0.001*) (Fig 3.5B&C). However, no correlation was seen between *Fv/Fm* and dry biomass of plants under control conditions (Fig 3.5A).

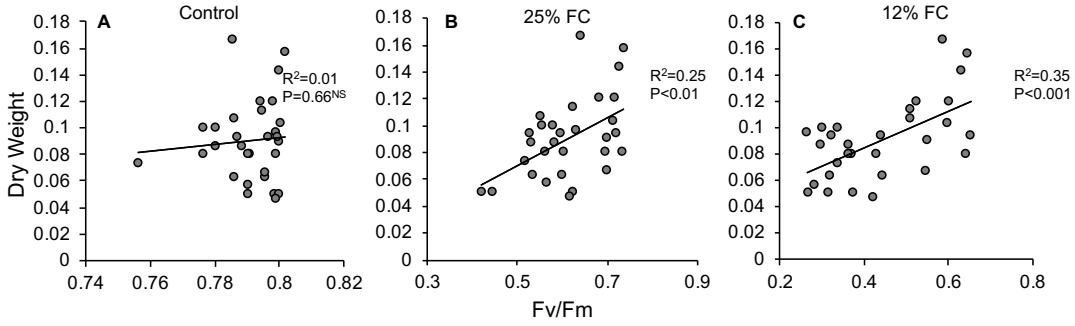

Figure 3.5 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and chlorophyll fluorescence (*Fv/Fm*) of 30 barley genotypes grown under control, 25% and 12% field capacity conditions

There was no significant correlation between stomatal conductance (Gs) and shoot dry weight for plants grown under control conditions (Fig 3.6A). However, a strong positive correlation was observed between Gs of plants grown under moderate (25% FC) and severe (12% FC) drought stress and the shoot DW (R^2=0.14; significant at P<0.05 and R^2=0.24, respectively; significant at P<0.01) (Fig 3.6B&C).

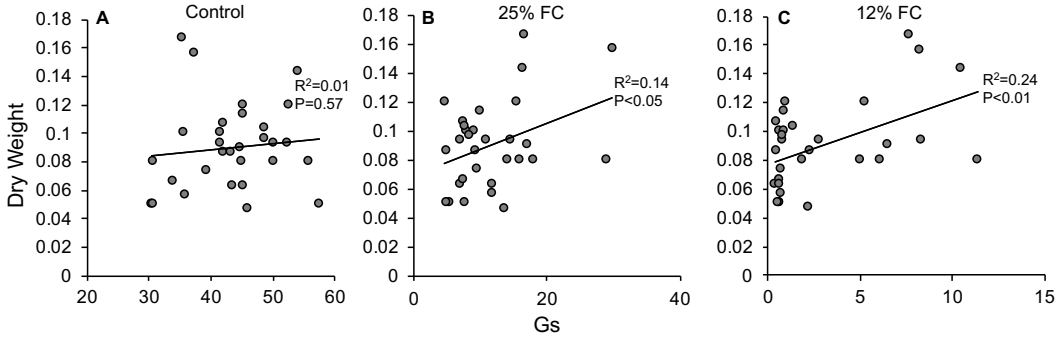

Figure 3.6 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and stomatal conductance (Gs) of 30 barley genotypes grown under control, 25% and 12% field capacity conditions

A strong positive correlation was found between shoot fresh weight and shoot dry weight for plants grown under control conditions (R^2=0.32 significant at *P<0.01*) (Fig 3.7A). A strong positive correlation was observed between shoot fresh weight of plant grown under moderate (25% FC) and severe (12% FC) drought stress and the shoot DW (R^2=0.20; significant at P<0.05 and R^2=0.43, respectively; significant at P<0.001) (Fig 7B&C).

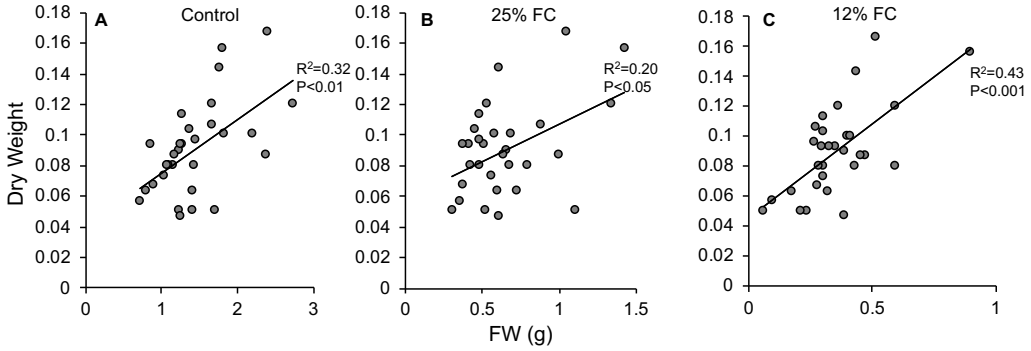

Figure 3.7 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and fresh weight (FW) of 30 barley genotypes grown under control, 25% and 12% field capacity conditions

A strong negative correlation was observed between shoot dry weight and relative water content of plants grown under control conditions and moderate drought stress (R^2=0.13; R^2=0.12 significant at *P<0.05*) (Fig 3.8A&B). However, a positive and significant (R^2=0.14 significant at *P<0.05*) relationship was found between relative water content and dry shoot weight of plants grown under 12% field capacity irrigation (Fig 3.8C).

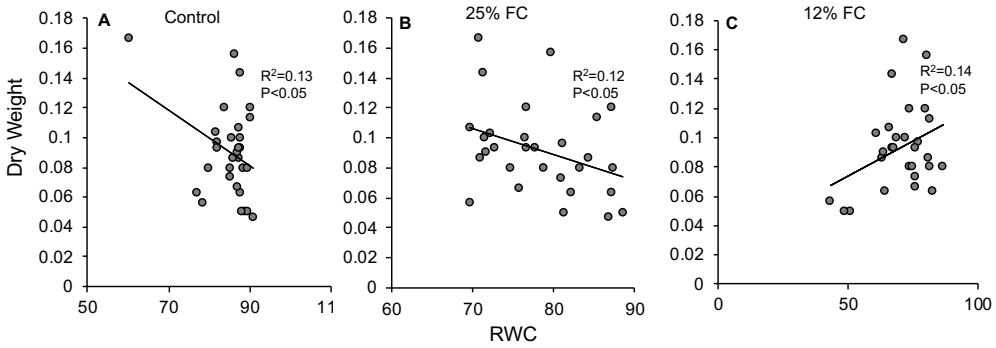

Figure 3.8 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and relative water content (RWC) of 30 barley genotypes grown under control, 25% and 12% field capacity conditions.

Table 3.11 The correlation matrix between shoot dry weight and major physiological characteristics of barley plants grown under control and deficit irrigation conditions. SPAD - leaf chlorophyll content; Fv/Fm – maximum photochemical efficiency of PSII; Gs- stomatal conductance; FW - fresh weight; RWC - relative water content

	SPAD			Fv/Fm			Gs			FW			RWC		
	Control	25%FC	12%FC	Control	25%FC	12%FC	Control	25%FC	12%FC	Control	25%FC	12%FC	Control	25%FC	12%FC
DW	0.02^{NS}	0.23**	0.47***	0.01^{NS}	0.25**	0.35***	0.01^{NS}	0.14*	0.24**	0.32**	0.20^{*}	0.43***	0.13*	0.12*	0.14*

*=P<0.05
**= P<0.01
***= P<0.0001
NS= Non-significant

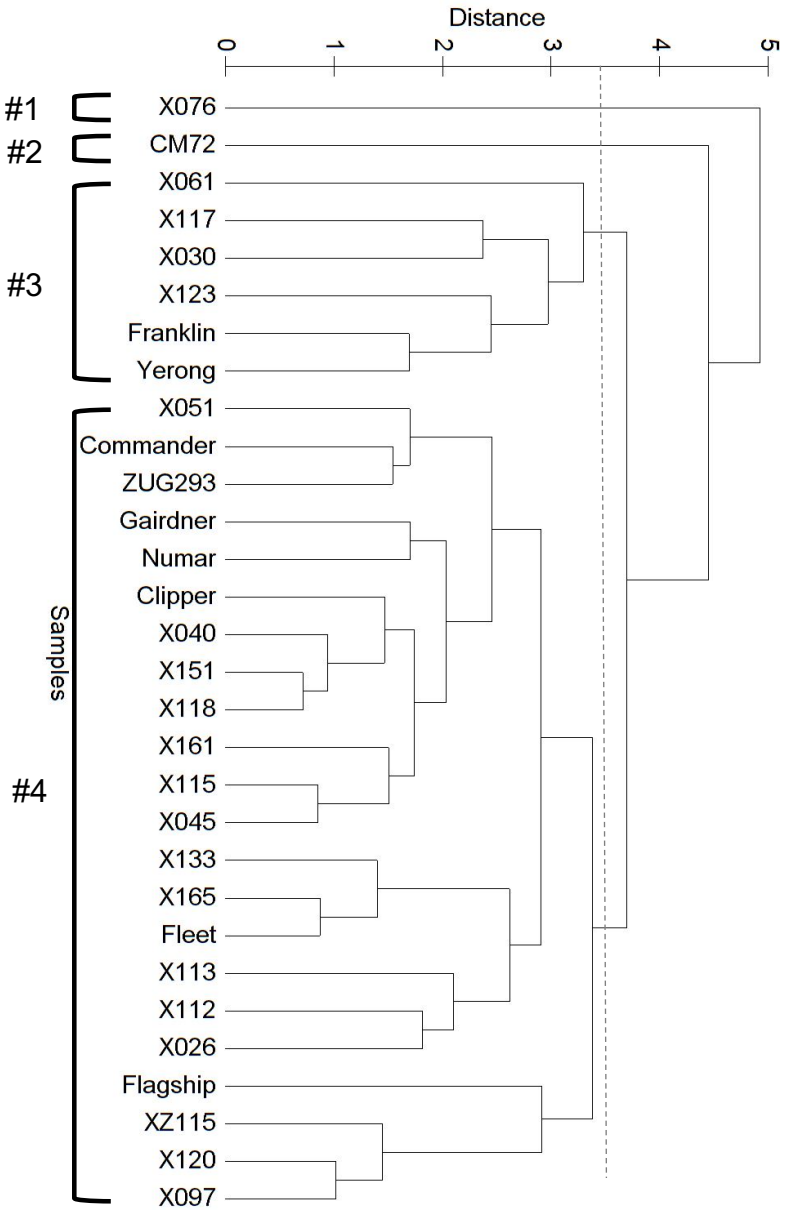

Figure 3.9 The hierarchical cluster analysis of 30 barley genotypes grown under control conditions. Plants are grouped into 4 groups based on chlorophyll content (SPAD), chlorophyll fluorescence (Fv/Fm), stomatal conductance (Gs), fresh weight (FW), dry weight (DW) and relative water content (RWC). The dendrogram shows fusion levels at which the groups join. The vertical dashed line represents the truncation into four genotype groups using Ward's agglomerative clustering algorithm.

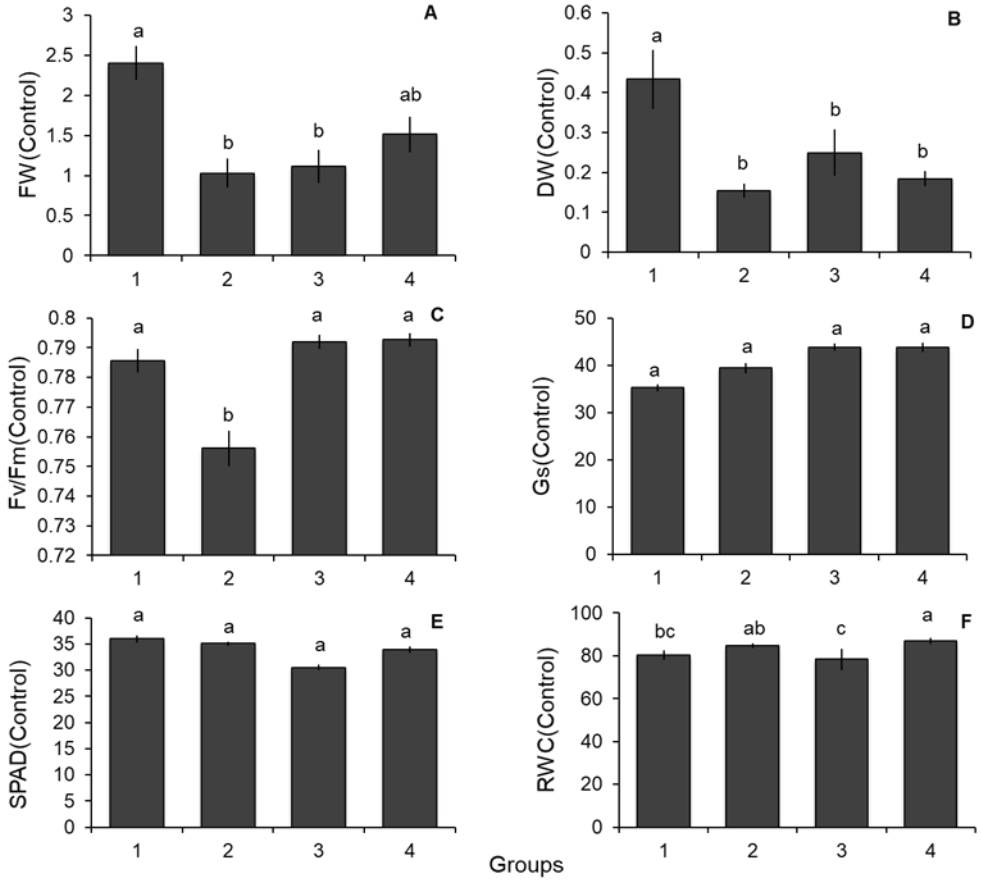

Figure 3.10 Comparison of the groups produced by cluster analysis in Fig 3.9, showing the differences between groups in fresh weight (A), dry weight (B), Fv/Fm (C), stomatal conductance (D), SPAD (E), relative water content (F), respectively. Different lowercase letters indicate the significance difference between clusters at *P<0.01*.

3.2.4 Cluster analysis for plant grown at full field capacity water content

Cluster analysis based on agronomical and physiological characteristics divided thirty barley genotypes grown under control conditions into four clusters (Fig 3.9). Mean values for each cluster are plotted in Fig 3.10. Cluster 1 have only X076 genotype. This genotype has higher fresh weight, dry weight and chlorophyll content and lowest stomatal conductance in irrigated conditions. Cluster 2 also consisted of only one genotype CM72. CM72 have lowest fresh weight, dry weight and *Fv/Fm.* Cluster 3 comprised of X061, X117, X030, X123, Franklin and Yerong. These genotypes exhibited highest *Fv/Fm* and stomatal conductance and very low chlorophyll content. Cluster 4 consisted of twenty two genotypes (X051, Commander, ZUG293, Gairdner, Numar, Clipper, X040, X151, X118, X161, X115, X045, X133, X165, Fleet, X113, X112, X026, Flagship, XZ115, X120, X097) and these genotypes had higher *Fv/Fm*, relative water content but low dry weight (Fig 3.10).

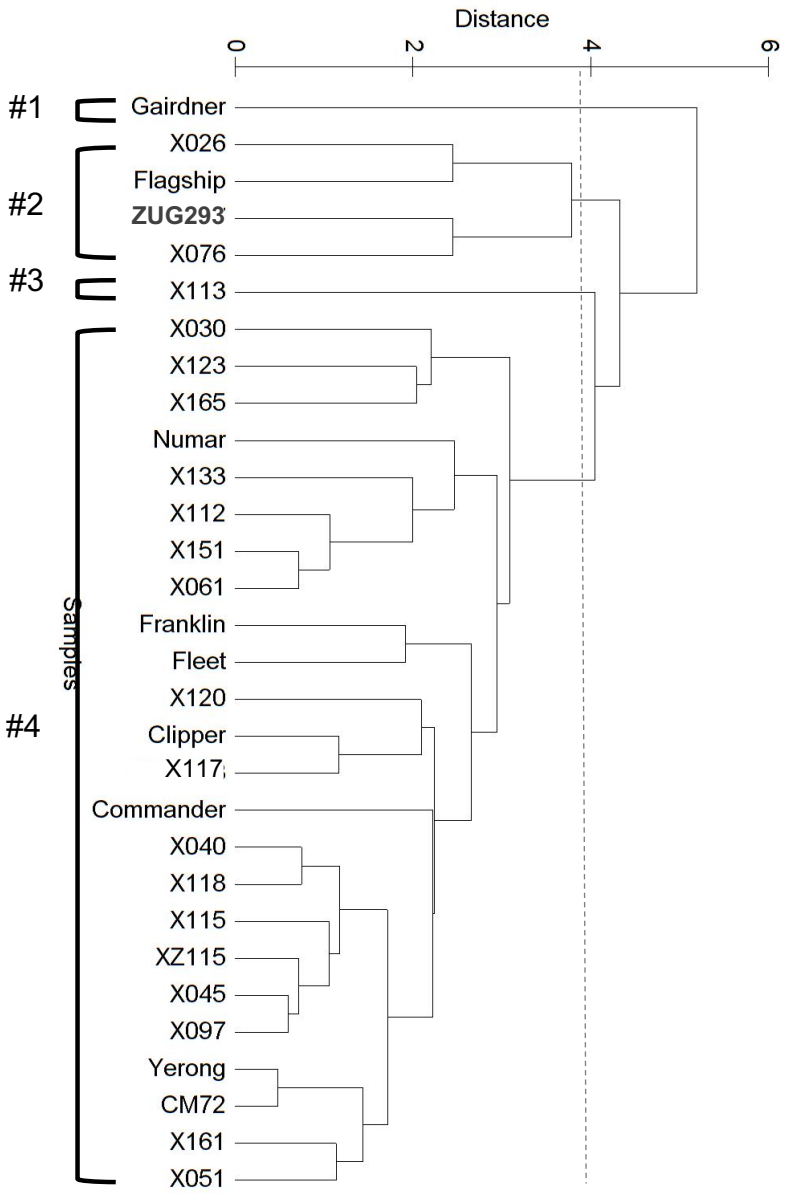

Figure 3.11 The hierarchical cluster analysis of 30 barley genotypes into 4 groups grown under moderate (25% field capacity) drought stress conditions. Plants are grouped based on chlorophyll content (SPAD), chlorophyll fluorescence (*Fv/Fm*), stomatal conductance (Gs), fresh weight (FW), dry weight (DW) and relative water content (RWC). The dendrogram shows fusion levels at which the groups join. The vertical dashed line represents the truncation into four genotype groups using Ward's agglomerative clustering algorithm

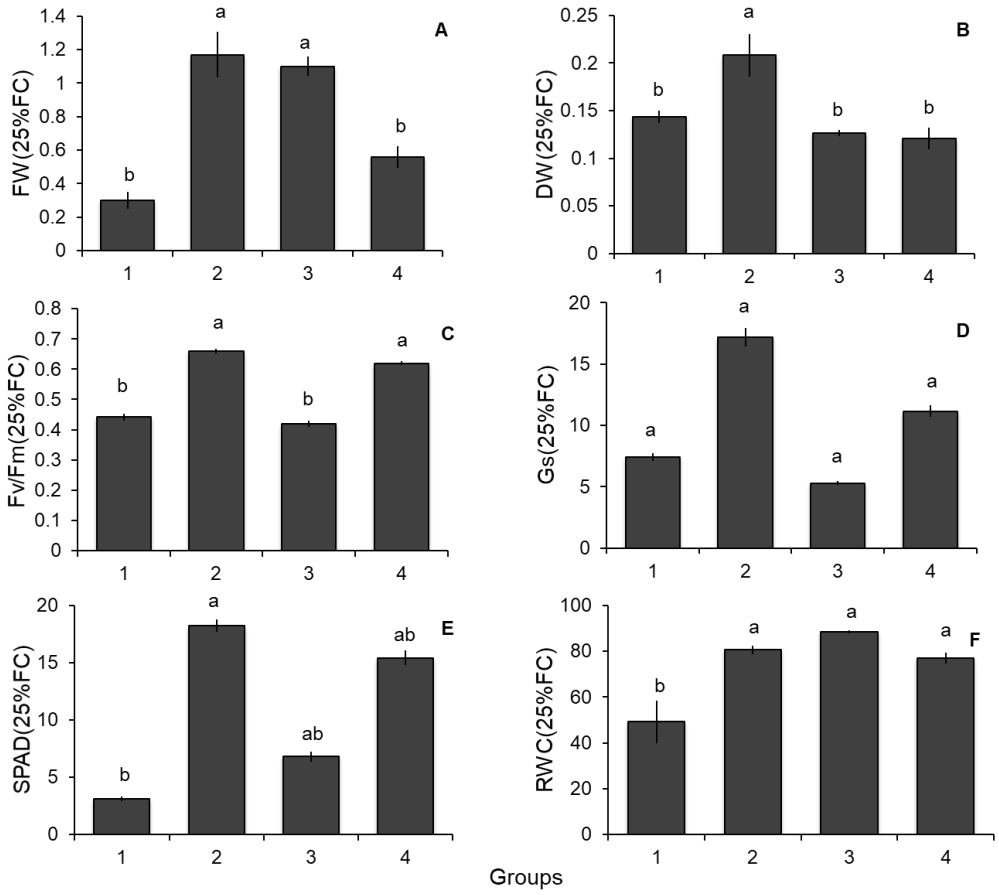

Figure 3.12 Comparison of the groups produced by cluster analysis in Fig 3.11 for plants grown under moderate (25% field capacity) stress conditions, showing the differences between groups in fresh weight (A), dry weight (B), *Fv/Fm* (C), stomatal conductance (D), SPAD (E), relative water content (F). Different lowercase letters indicate the significance difference between clusters at $P<0.01$.

3.2.5 Cluster analysis for plants grown at 25% field capacity water content

Cluster analysis based on agronomical and physiological traits divided thirty barley genotypes grown under moderate (25% field capacity) conditions into four clusters (Fig 3.11). Cluster 1 consisted of only one genotype Gairdner; this genotype has lowest chlorophyll content, relative water content and fresh weight (Fig 3.12). Cluster 2 comprised of X026, Flagship, X117 and X076; these genotypes had highest fresh weight, dry weight, *Fv/Fm,* chlorophyll content and stomatal conductance. The cluster 2 can be referred as the most tolerant cluster among all four. Cluster 3 had only one genotype X113; this genotype had least *Fv/Fm*, stomatal conductance but highest relative water content. Cluster 4 comprised of X030 to X051 (Fig 3.11) genotypes; these genotypes have intermediate values for all parameters but in terms of dry weight these genotypes had the least dry weight (Fig 3.12).

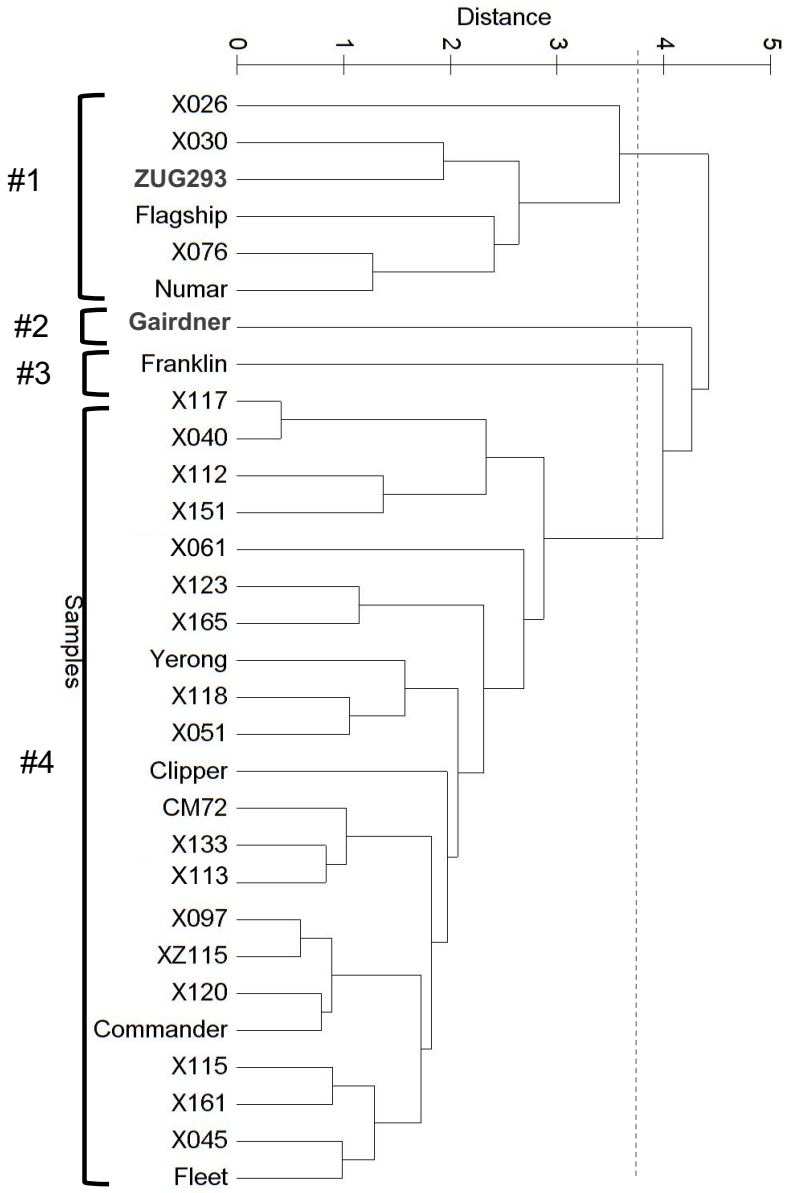

Figure 3.13 The hierarchical cluster analysis of 30 barley genotypes into 4 groups grown under severe (12% field capacity) drought stress conditions. Plants are grouped based on chlorophyll content (SPAD), chlorophyll fluorescence (*Fv/Fm*), stomatal conductance (Gs), fresh weight (FW), dry weight (DW) and relative water content (RWC). The dendrogram shows fusion levels at which the groups join. The vertical dashed line represents the truncation into four genotype groups using Ward's agglomerative clustering algorithm.

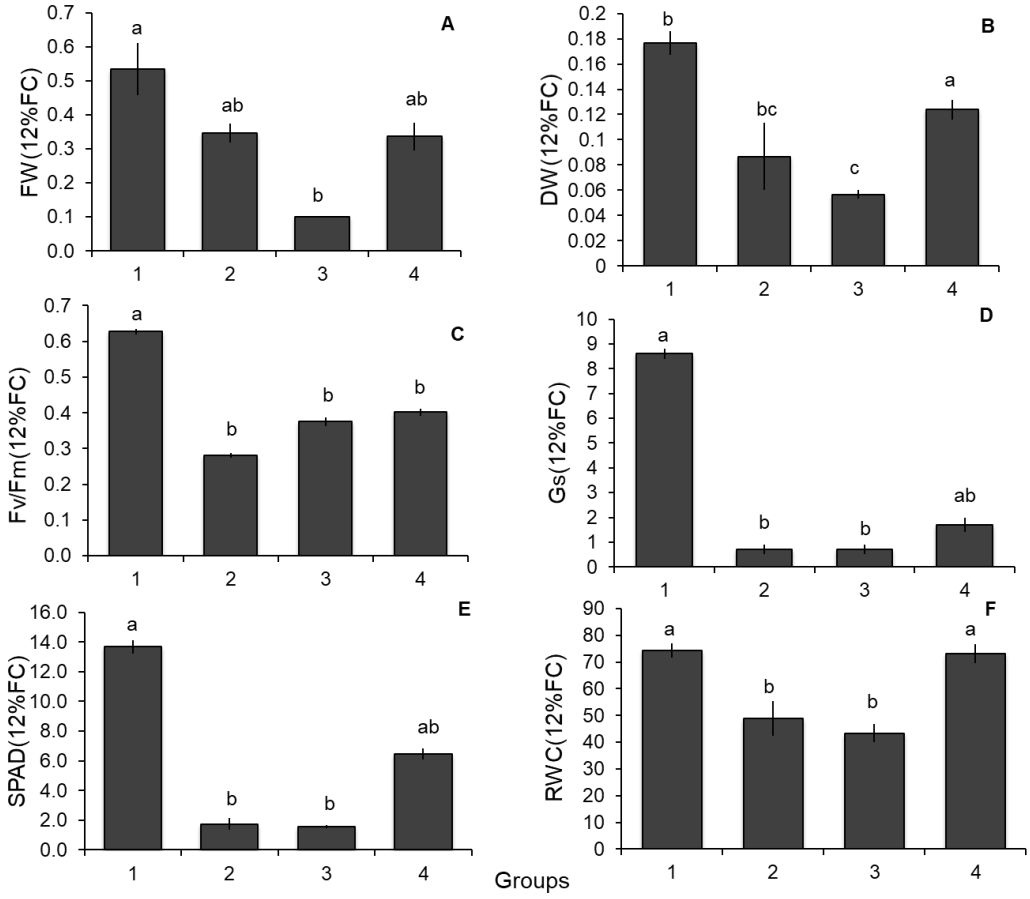

Figure 3.14 Comparison of the groups produced by cluster analysis in Fig 13 for plants grown under severe (12% field capacity) stress conditions, showing the differences between groups in fresh weight (A), dry weight (B), Fv/Fm (C), stomatal conductance (D), SPAD (E), relative water content (F), respectively. Different lowercase letters indicate the significance difference between clusters at $P<0.01$

3.2.6 Cluster analysis for plants grown at 12% field capacity water content

Cluster analysis based on physiological and agronomical traits divided thirty barley genotypes grown under severe stress (12% FC) conditions into four clusters (Fig 3.13). The first cluster encompassed the genotypes X026, X030, ZUG293, Flagship, X076 and Numar; these genotypes had highest fresh weight, dry weight, *Fv/Fm*, stomatal conductance, chlorophyll content and relative water content (Fig 3.14). Cluster 1 can be referred as the tolerant group among all. Cluster 2 had only one (Gairdner) genotype and cluster 3 also had only one (Franklin) genotype. Both of these clusters showed lowest *Fv/Fm*, chlorophyll content, stomatal conductance, relative water content and dry weight. The genotypes in cluster 2 and 3 can be referred as drought sensitive. Cluster 4 gathered together all the genotypes from X113 to Fleet; these genotypes have moderate values for all parameters and can be referred as moderate tolerant group.

3.3 Discussion

3.3.1 Genetic diversity of barley

Barley is one of the most stress-tolerant crops with a large and diversified genetic pool (Gurel et al., 2016; Munoz-Amatriain et al., 2014) including numerous landraces adapted to arid and semi-arid environments (Kosova et al., 2014). Wild barley germplasms are a rich source of genetic diversity and their use has been considered potentially beneficial for the improvement of drought tolerance (Zhao et al., 2010). Over the past 100 years, extensive cultivation, breeding and selection have substantially modified barley germplasm. Such ample genetic diversity facilitate the breeders to identify tolerant genotypes and use the breeding programs to combine stress tolerance with elevated yield potential (Von Bothmer et al., 2003). A large number of barley genotypes were used in this study, collected from different origins grown under drought stress conditions. Our results based on both screening experiments for drought tolerance efficiently described genetic diversity in wild and cultivated barley germplasm which is in good agreement with previous findings in which wild and cultivated barley exhibited high genetic variability for drought tolerance as well as the differential responses of genotypes across different environments (Barati et al., 2018; Chen et al., 2005; Nazari and Pakniyat, 2008). In the first experiment, tolerance evaluation was made on drought damage index. After the cease of water application, the leaves started to show the chlorotic symptoms and with the severity of water stress condition, these chlorotic leaves turned into necrotic and ultimately caused leaf senescence (Hailemichael et al., 2016). The possible reason for this is due to lack of available water and nutrients mainly nitrogen, potassium, phosphorus, sodium and magnesium for plants to uptake for their normal plant growth (Wong, 2006). We selected four genotypes with lowest drought damage index > 6.5 as the most drought tolerant genotypes (Numar, ZUG293, Flagship, and X026) (Fig 3.1). One possible reason of their tolerance could be their origin as Numar, ZUG293 and Flagship were originated from arid or semi-arid areas of California, Sudan and South Australia respectively (see details Tab 2.1). Similarly, the drought tolerant wild barley genotype X026 belongs to Tibet, China which is well known for its harsh environments and therefore genotypes grown in Tibet have high tolerance to abiotic stresses (Cai et al., 2013). According to Tyagi et al. (2011), a strong correlation was found between habitat and drought stress tolerance in many Jordan and Israel barley varieties. These genotypes might adapt some morphological traits to reduce the

symptoms of chlorosis and necrosis such as longer roots that facilitate better access to water and nutrients mainly N, a soluble nutrient that tends to leach into the deeper layers of the soil (Wasson, A.P. et al., 2012). Also, these genotypes could have ability to maintain relatively high water through reduced evapo-transpiration surface (leaf area) (Dossa et al., 2017). Leaf rolling is another strategy to adapt water stress as rolled leaves could transpire 41 per cent less water than the unrolled ones (Courtois et al., 2000). In the pot experiment where the genotypes were exposed to three different drought irrigation regimes, the tolerance was evaluated on shoot biomass. Based on cluster analysis (Fig 3.13 & 3.14), three cultivated (Numar, Flagship and ZUG293) and three wild (X076, X026 and X030) genotypes were identified as drought tolerant. All the tolerant genotypes showed relatively less reduction in shoot fresh and dry weight under severe drought stress. They maintained high relative water content and exhibited less reduction in photosynthetic traits such as SPAD, *Fv/Fm* and stomatal conductance. The results are in line with results of the first experiment as the genotypes with low damage index accumulated high shoot biomass and improved other physiological characteristics. However, some genotypes (X030 and X076) showed very high drought damage index but accumulated high biomass. Interestingly, Gairdner and Franklin were grouped as drought susceptible genotypes in both the screening experiments as these genotypes exhibited high drought damage index and accumulated lowest shoot biomass under severe drought conditions (12% field capacity irrigation). Gairdner and Franklin are commercial varieties grown in high rainfall areas in Australia. Therefore, these varieties are not tolerant to water deficit conditions. Gairdner and Franklin are also sensitive to other abiotic stresses such as salinity and waterlogging (Angessa et al., 2017; Chen et al., 2007; Zhang et al., 2015). All wild barley genotypes were selected as drought tolerant or moderate drought tolerant based on their biomass under severe drought stress (Fig 3.13). We can say that wild genotypes are no doubt a great source of genetic variability, but genetic variation also existed in cultivated barley genotypes. The genetic diversity of barley enabled us to select a range of cultivars to investigate mechanisms underlying drought tolerance.

3.3.2 Assessing suitability of various screening approaches

Different methods were adopted to induce drought stress and evaluated the tolerance based on drought damage index and biomass. We also observed agronomical and physiological responses to drought stress in thirty barley genotypes including wild and cultivated. Though trends in the different responses of these genotypes to drought stress were fairly consistent while using both screening techniques, there were few genotypes that showed some dissimilarities between methods. Visual evaluation based on drought damage index provided a simple and feasible technique to measure the tolerance to water stress. The irrigation was withheld when the seedlings were 14 days old and were exposed to progressive drought for 49 days. Data collection was just based on counting total number of chlorotic and necrotic leaves as well as total number of leaves at three time points. Sampling did not require any special equipmental expertise hence, not a laborious screening technique. Screening of hundreds and thousands plants can be done using visual scoring. A major limitation in this method could be unreliability caused by biasness or human error as reported in the past when visual scoring to plants was given by several experts (Kaya and Taner, 2016; Li et al., 2014). Therefore, this kind of human error could be the main reason for high damage scoring index given to some genotypes as they accumulated high biomass in the second experiment. The researchers in the past working with small pots and complete withholding water considered the fact that completely stopping irrigation caused rapid drying and might prevented the plant from adjusting to the new conditions (Negin and Moshelion, 2017). Therefore, we can recommend breeders to conduct screening by using relatively large tanks under which plants are more gradually exposed to water deficits, thus providing more uniform background and increasing reliability of the procedure.

Pot experiment performed under three different water deficit regimes was difficult to handle as compared to the former technique. This method may have two possible limitations. The first is, maintaining field capacity on daily basis which is a labor-intensive and time- consuming job and involved weighing and adding appropriate water to maintain the required field capacity. In the pot experiment, water could be rapidly lost from the soil therefore roots could hardly contribute to maintain the water uptake (Iseki et al., 2018), this could be the second main drawback in this methodology. These limitations highlighted the difficulty of collecting data in this experiment. Hence, this method can be design for a limited range of plants and could not recommended

for screening on a large scale. However, the convenience of this method is the accuracy to which the drought level is controlled.

The suitability of various physiological and agronomical indices was assessed as proxies for barley drought tolerance for high-throughput screening of barley germplasm. SPAD and *Fv/Fm* values were positively correlated to shoot dry weight under drought stress. Both chlorophyll content and chlorophyll fluorescence have been suggested as selection criteria for drought tolerance and for screening a large number of barley genotypes for breeding (Filek, M. et al., 2015; Guo et al., 2008; Hasanuzzaman et al., 2017; Sharma et al., 2015). The measurements were quick, noninvasive and can generate a large amount of data in very short time. Stomatal conductance and leaf water content both measures plant water consumption. Both of these characteristics exhibited significant correlation with biomass (Arjenaki et al., 2012; Farooq et al., 2009b; Jaleel et al., 2008; Vanaja et al., 2015). However, measuring stomatal conductance of plants grown under severe drought stress conditions (12% field capacity) was very difficult as stomata were closed under stress and leaves were rolled and reduced in size (Spreer et al., 2006; Stiller et al., 2003). Relative water content could be a good indicator for screening genotypes, but it is time consuming and difficult for screening a large number of genotypes. Plant dry biomass provide reliable tolerance information as most of the tolerant genotypes with lowest drought damage index accumulated relatively more dry weight under drought stress conditions. However, the genotypes with highest damage index accumulated lowest shoot dry weight.

3.3.3 Genotypes with high stomatal conductance maintained high relative water content

Results of cluster analysis (group #1, Fig 3.14) revealed that drought tolerant genotypes had greater stomatal conductance and maintained higher relative water content under severe water stress compared to drought sensitive ones (Fig 3.14). These high stomatal conductance rates indicate that these genotypes did not closed their stomata as earlier as other genotypes, maintaining increased carbon assimilation /photosynthetic rate and thus growth. Interestingly, despite having higher Gs values, drought tolerant genotypes were still able to maintain high relative water content in drought stress, indicating greater osmotic adjustment. Plants adapts different strategies to maintain leaf water content under water deficit either by closing stomata or by

osmotic adjustment. Osmotic adjustment is one of the important mechanism which is effective in sustaining turgor and relative water content in plants (Blum, 2017). Under drought stress, plants readjust their osmotic potential either by enhanced uptake of inorganic ions or by de novo synthesis of compatible solutes. Several plant species accumulate organic solutes mainly sugars and proline in response to water stress (Liu et al., 2015). However, as both the severity of the drought and osmotic stress increase, this drought induced hyperosmotic stress slow down the production of compatible solutes due to their high metabolic energy cost (10 times higher than inorganic ion uptake) (Raven, 1985; Shabala and Lew, 2002). Therefore, plants use inorganic ions uptake (mainly K^+, Na^+, and Cl^-) to provide fast and efficient osmotic adjustment to maintain high relative water content (Shabala and Shabala, 2011).

The results of the present study revealed that genetic variation exists in wild and cultivated barley in response to drought stress tolerance. Numar, Flagship, ZUG293, and X026 were found to be most drought tolerant with high biomass. At the same time, Gairdner and Franklin were selected as most drought susceptible genotypes. These genotypes are therefore recommended for mapping DH population, to reveal the QTLs responsible for drought stress tolerance in barley. Though, both screening techniques were justifying the same results, visual evaluation of plants based on drought damage index was more simple and recommended; the use of large tanks is also highly recommended. The results obtained in this study underline the important role of various adaptive mechanisms in protecting plant during water deficit conditions and may be important for the selection for drought tolerance.

Chapter 4. Genotypic variation of drought tolerance in bread and durum wheat

4.1 Introduction

Wheat is the major staple food which provides approximately 20% of daily calories and protein for 4.5 billion people around the world (Shiferaw et al., 2013). Currently, two major wheat species, hexaploid bread wheat (*Triticum aestivum*; 2n = 6x = 42) and tetraploid durum wheat (*Triticum durum*; 2n = 4x = 28), are commercially important. Bread wheat is representing more than 90% and durum around 5% of total wheat production (Monneveux et al., 2012). Wheat ranks first in terms of harvested area (223.67 million hectares in 2016) and is the second most produced crop with a global production of 735.3 million tons in 2016 (USDA, 2017). While an urgent need to increase food production is necessary to match rapid growing world population (Naeem et al., 2015), these efforts are hampered by various biotic and abiotic stresses affecting crop production.

Among various abiotic stresses, water deficit is the most devastating factor and is one of the leading constraints to wheat production globally. According to data published from 1980 to 2015, the yield reduction in wheat caused by water deficit ranged from 21% to 40% across the globe (Daryanto et al., 2016). Drought tolerant wheat varieties are the ultimate means of safeguarding the crop against adverse effects of the stress.

It is generally accepted that bread wheat is known to possess higher drought tolerance compared with durum wheat genotypes (Allahverdiyev and Huseynova, 2017; Allahverdiyev, 2015; Marti and Slafer, 2014). However, some studies showed that durum wheat showed comparatively less reduction in the biomass production under drought than bread wheat (Ayed et al., 2017; Saleem, 2003). The reason for such controversy may be the difference in the severity and duration of the stress, and the intraspecific genetic variability within each group.

The progress in breeding for drought tolerance is also significantly handicapped by the lack of consensus on the best phenotyping method. Visual scoring based on leaf senescence including leaf chlorosis and necrosis are often used as a phenotypic marker to evaluate plant tolerance to stresses such as salinity and water logging (Negrao et al., 2017; Zeng et al., 2013). Until now this methodology has not been applied to wheat in the context to drought stress. Variation in physiological traits, including chlorophyll content, stomatal conductance relative water content, biomass, quantum yield of PSII (*Fv/Fm*), are mostly associated with wheat's response to drought stress (Pour-Aboughadareh et al., 2017; Saeidi et al., 2015). Under mild to moderate drought stress, stomatal closure (causing reduced leaf internal CO_2 concentration, or Ci) is the major reason for reduced photosynthesis (Flexas et al., 2008). This leads to less assimilate production and thus lower yields. Severe drought stress further inhibits photosynthesis by decreasing chlorophyll content (mainly the result of damage to chloroplasts caused by reactive oxygen species, affecting chlorophyll components, and by damaging the photosynthetic apparatus (Iturbe-Ormaetxe et al., 1998). Therefore, chlorophyll content provides a key indicator of the photosynthetic capacity (Cannella et al., 2016; Houborg et al., 2015). Another valuable index is chlorophyll fluorescence and, specifically, the maximum quantum efficiency of light harvesting in PSII in dark adapted leaves used for evaluating plant drought tolerance (Paknejad et al., 2007; Sharma et al., 2015). Being rapid and non-invasive, the chlorophyll fluorescence measuring technique has been widely accepted as an effective and reliable diagnostic tool for high-throughput assessments of plant germplasm for drought tolerance (Guo et al., 2008; Hasanuzzaman et al., 2017). Similar to leaf water potential, leaf RWC gives a strong indication of the plant's response to drought stress conditions (Chowdhury et al., 2017); yet RWC has been shown to be a more stable parameter than leaf water potential (Sade et al., 2012; Sade et al., 2009).

Another interesting question is whether wheat species employ similar mechanisms to deal with drought stress as compared to barley. Barley is classified as drought- and salt stress tolerant species (Li et al., 2007) and was shown to have higher yield potential relative to wheat under drought conditions (Woldeamlak et al., 2006). However, Jamieson et al. (1995) concluded that yield response was mostly the same in wheat and barley under water deficit.

The objectives of the study were three-fold: (1) to determine the extent of genotypic variation in drought tolerance among bread and durum wheat germplasm to identify promising lines for breeders and growers; (2) to understand the physiological basis of drought tolerance in wheat; and (3) to reveal the differences in drought adapting strategies between wheat and barley.

4.2 Results

4.2.1 Glasshouse screening based on visual scoring

Drought tolerance of bread and durum wheat germplasm was evaluated on the basis of the total number of leaves, number of chlorotic and necrotic leaves and visual scoring. Variety Xinong223 showed the highest number of leaves (13.75 ± 0.25) in third and fifth week after drought was imposed. Tainong292 produced the highest number of leaves per plant (13.50 ± 0.25) at the end of seventh week (Table 4.1). In contrast to this, variety Kord Cl Plus had the least number of leaves (6.75 ± 0.48) after three and five weeks of drought, and variety Pobeda produced the least number of total leaves at the end of seventh week (7.00 ± 0.25).

Table 4.1 Total number of leaves including chlorotic and necrotic leaves at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
Albidum24	7.00±0.41	7.50±0.29	7.50±0.29
Onohoiskaja4	7.75±0.25	7.75±0.25	7.75±0.25
Surhak Mestnyj	8.75±0.25	7.75±0.25	7.75±0.25
Pobeda	7.75±0.25	7.75±0.25	7.00±0.25
Kord Cl Plus	6.75±0.48	7.25±0.48	7.25±0.48
Mahon Demias	8.75±0.25	8.75±0.25	7.75±0.25
Preto Amarelo	8.50±0.29	8.00±0.24	7.50±0.29
Emai19	9.25±0.25	9.25±0.25	9.25±0.25
Xiangmai25	9.75±0.25	9.75±0.25	9.75±0.25
Liangxing99	7.50±0.29	7.50±0.29	7.50±0.29
Zhengmai9023	8.75±0.25	8.75±0.25	8.00±0.25
Ningmai17	8.50±0.29	8.50±0.29	8.50±0.29
Xinong2000	8.50±0.29	8.50±0.29	8.50±0.29
Xinong223	13.75±0.25	13.75±0.25	13.00±0.21
Linyuan8	9.75±0.25	9.75±0.25	9.75±0.25
Yumai57	8.50±0.50	8.50±0.50	8.50±0.50
Huanong5	12.75±0.25	11.75±0.22	11.75±0.21
Huaimai16	9.75±0.25	9.75±0.25	9.00±0.25
Zhoumai16	9.50±0.50	9.00±0.24	9.00±0.29
Tainong292	13.5±0.50	13.0±0.50	13.5±0.50

The number of chlorotic leaves varied between 1.75 ± 0.25 and 0.25 ± 0.25 in the third week. With the increase in stress duration (fifth and seventh week), the chlorotic leaves found to be decreased as the new leaves did not develop and chlorotic leaves possibly became necrotic by the period of five and seven weeks of drought. Hence, the number of chlorotic leaves ranged between 1.25 ± 0.25 to 0.25 ± 0.25 at fifth week and varied between 0.75 ± 0.25 to 0.25 ± 0.25 at seventh week (Table 4.2).

Table 4.2 Total number of chlorotic leaves at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
Albidum24	0.25±0.25	0.25±0.25	0.50±0.29
Onohoiskaja4	0.75±0.25	0.50±0.29	0.75±0.25
Surhak Mestnyj	0.50±0.29	0.50±0.29	0.25±0.25
Pobeda	1.75±0.25	0.50±0.29	0.25±0.25
Kord Cl Plus	0.75±0.25	0.00±0.00	0.50±0.29
Mahon Demias	0.25±0.25	0.25±0.25	0.25±0.25
Preto Amarelo	1.75±0.25	1.25±0.25	0.75±0.25
Emai19	1.25±0.25	0.50±0.29	0.50±0.29
Xiangmai25	0.50±0.29	0.50±0.29	0.50±0.29
Liangxing99	0.50±0.29	0.25±0.25	0.25±0.25
Zhengmai9023	1.00±0.00	0.50±0.29	0.50±0.29
Ningmai17	0.50±0.29	0.25±0.25	0.25±0.25
Xinong2000	0.50±0.29	0.25±0.25	0.25±0.25
Xinong223	0.25±0.25	0.75±0.25	0.50±0.29
Linyuan8	0.50±0.29	0.50±0.29	0.50±0.29
Yumai57	0.50±0.29	0.25±0.25	0.25±0.25
Huanong5	1.00±0.00	0.25±0.25	0.50±0.29
Huaimai16	0.50±0.29	0.50±0.50	0.50±0.50
Zhoumai16	0.75±0.25	0.75±0.25	0.75±0.25
Tainong292	1.25±0.25	0.50±0.29	0.50±0.29

Drought tolerance was also assessed by the total number of necrotic leaves produced by drought (Table 4.3). After three weeks of withholding the irrigation, the number of necrotic leaves varied between 1.50 ± 0.29 (Preto Amarelo) and 0.25 ± 0.25 (Xinong2000). At 5th week of drought, the number of necrotic leaves ranged between 8.00 ± 0.41 to 3.25 ± 0.25. The maximum number of necrotic leaves was exhibited by Huanong5 and the minimum number of necrotic leaves was found in Kord Cl Plus. By the end of 7th week, the average number of necrotic leaves varied between 8.75 ± 0.23 and 3.50 ± 0.29. The maximum number of necrotic leaves were produced by Xinong223, and the minimum number of necrotic leaves was found in Liangxing99 followed by Yumai57 (4.00 ± 0.41).

Table 4.3 Total number of necrotic leaves at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
Albidum24	1.25±0.25	4.75±0.48	6.25±0.75
Onohoiskaja4	1.25±0.25	3.75±0.25	5.75±0.75
Surhak Mestnyj	0.50±0.29	5.50±0.29	6.25±1.03
Pobeda	0.75±0.25	5.75±0.25	7.25±0.48
Kord Cl Plus	0.50±0.29	3.25±0.25	7.25±0.48
Mahon Demias	0.75±0.25	6.25±0.48	5.00±0.40
Preto Amarelo	1.50±0.29	5.00±0.41	6.25±0.75
Emai19	0.75±0.25	4.75±0.48	6.50±0.87
Xiangmai25	1.00±0.00	5.50±0.29	5.75±0.25
Liangxing99	0.75±0.25	3.50±0.29	3.50±0.29
Zhengmai9023	1.00±0.41	7.00±0.25	7.75±0.25
Ningmai17	0.75±0.25	5.50±0.29	5.50±0.29
Xinong2000	0.25±0.25	5.75±0.25	5.75±0.48
Xinong223	0.75±0.48	6.25±0.25	8.75±0.23
Linyuan8	0.75±0.25	7.25±0.25	6.50±0.29
Yumai57	0.50±0.29	4.00±0.41	4.00±0.41
Huanong5	0.75±0.25	8.00±0.41	7.50±0.29
Huaimai16	0.50±0.29	4.50±0.29	4.50±0.29
Zhoumai16	0.50±0.29	5.75±0.25	6.00±0.41
Tainong292	0.75±0.48	6.75±0.25	6.75±0.25

Visual scoring for drought damage was assigned to all the genotypes after 3rd, 5th and 7th week of drought imposed (0 = no visual symptoms of stress; 10 = all plants are dead) (Table 4.4). In the third week the visual scoring varied between 2.00 ± 0.00 and 1.00 ± 0.00. The highest score was found in genotype Preto Amarelo and the lowest in cultivar Albidum24, Xinong2000, Yumai57 and Huaimai16. At week 5 of the stress, drought damage index ranged between 8.25 ± 0.25 and 5.00 ± 0.25, and at week 7 the highest damage score was 9.50 (for genotypes Pobeda and Liangxing99) and the lowest was 5.50 ± 0.28 (for Mahon Demias) (Table 4.4).

Table 4.4 Drought damage index of the genotypes at 3rd, 5th and 7th week after drought imposed. Data are mean ± SEM (n = 4)

Genotypes	3 weeks	5 weeks	7 weeks
Albidum24	1.00±0.00	5.75±0.25	6.25±0.50
Onohoiskaja4	1.25±0.25	7.75±0.25	8.75±0.48
Surhak Mestnyj	1.25±0.25	7.75±0.25	9.00±0.58
Pobeda	1.25±0.25	8.00±0.00	9.50±0.29
Kord Cl Plus	1.25±0.25	7.25±0.48	8.00±0.64
Mahon Demias	1.25±0.25	5.00±0.25	5.50±0.28
Preto Amarelo	2.00±0.00	7.25±0.25	8.75±0.63
Emai19	1.25±0.25	6.75±0.25	8.50±0.87
Xiangmai25	1.25±0.25	6.00±0.41	7.00±0.41
Liangxing99	1.25±0.25	8.25±0.25	9.50±0.25
Zhengmai9023	1.50±0.29	7.50±0.00	9.00±0.29
Ningmai17	1.50±0.29	7.00±0.00	7.00±0.41
Xinong2000	1.00±0.00	7.50±0.29	6.75±0.48
Xinong223	1.25±0.25	6.50±0.29	7.25±0.63
Linyuan8	1.25±0.25	7.50±0.29	8.25±0.48
Yumai57	1.00±0.00	5.25±0.25	6.25±0.25
Huanong5	1.25±0.25	7.00±0.41	7.75±0.48
Huaimai16	1.00±0.00	5.75±0.25	6.25±0.25
Zhoumai16	1.50±0.29	7.00±0.00	8.00±0.58
Tainong292	1.75±0.25	5.25±0.25	6.00±0.00

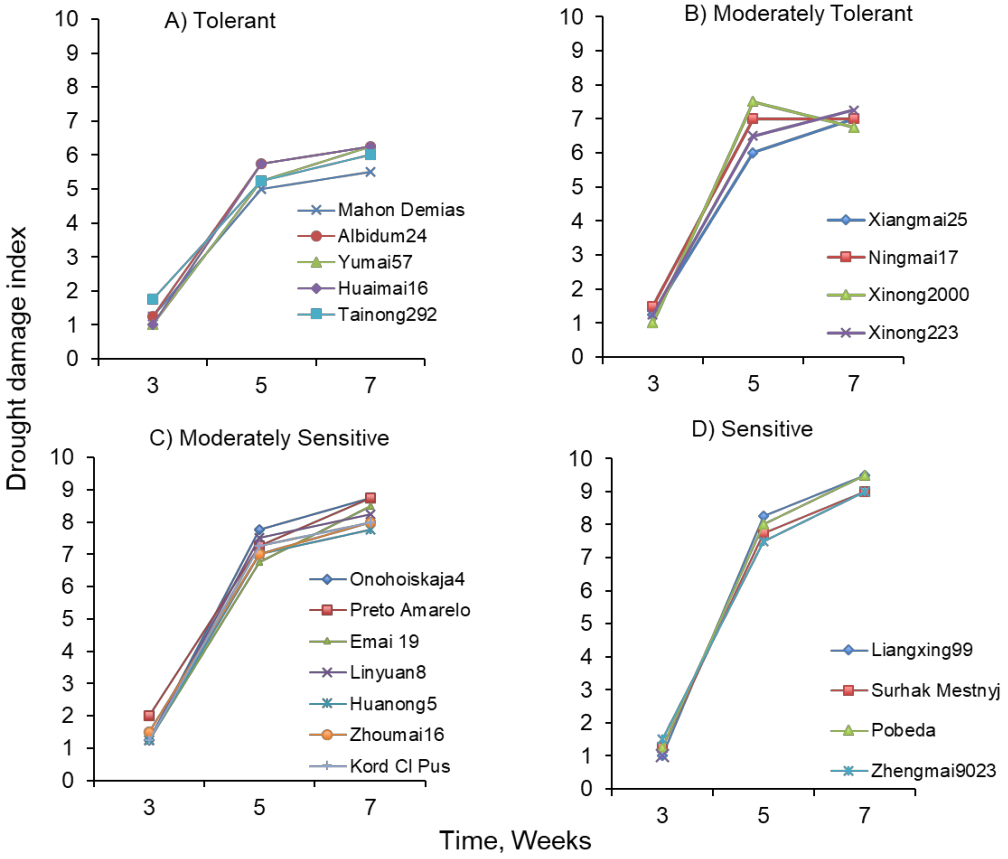

Figure 4.1 Four major groups were distinguished based on drought damage index: A, tolerant (DDI=5.5-6.25); B, moderately tolerant (DDI=6.50-7.25); C, moderately sensitive (DDI=7.5-8.75); D, sensitive (DDI=9-9.50)

Based on obtained visual scoring for drought damage, all wheat genotypes were clustered into four groups. The tolerant cluster contained varieties Mahon Demais, Albidum24, Yumai57, Huaimai16, Tainong292 (with the drought damage index 5.5-6.5). Moderately tolerant cluster included cultivars Xiangmai25, Ningmai17, Xinong2000, Xinong223 (damage index between 6.50 and 7.25); moderately sensitive cluster contained genotypes Onohoiskaja4, Preto Amarelo, Emai19, Linyuan8, Huanong5, Zhomai16, Kord Cl Plus (damage index between 7.5 and 8.75); and a sensitive group included genotypes Linxing99, Surhak Mestnyj, Pobeda, Zhengmai9023 (damage index over 9) (Fig 4.1).

4.2.2 Analysis of physiological characteristics and biomass

The second glasshouse screening experiment was performed on the basis of physiological characteristics and biomass. The experiment was carried out in pots filled with a potting mixture under controlled irrigation conditions: full irrigation (control); and two water deficit irrigations (25% and 12% of full field capacity).

Under irrigated conditions, SPAD values differed significantly between genotypes, ranging between 39.1 ± 0.57 to 20.6 ± 0.19 (Table 4.5). The highest SPAD value was for genotype Tainong292, and the lowest – for Albidum24. Under 25% field capacity irrigation, chlorophyll content (SPAD) greatly reduced, ranging between 25.9 ± 0.39 and 8.1 ± 0.19. Genotype Zhengmai9023 had the maximum chlorophyll content whereas Kord Cl Plus had the lowest. SPAD values ranged between 14.0 ± 0.32 (Zhengmai9023) and 1.8 ± 0.14 (Preto Amarelo) under severe drought stress (12% field capacity irrigation regime).

Table 4.5 Genotypic variability in chlorophyll content (SPAD) under controlled irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
Albidum24	20.6±0.19	17.5±0.21	12.6±0.16
Onohoiskaja4	36.4±0.60	12.9±0.42	6.1±0.37
Surhak Mestnyj	34.8±0.20	17.2±0.32	6.5±0.19
Pobeda	34.2±0.38	11.6±0.28	3.6±0.16
Kord Cl Plus	35.8±1.43	8.1±0.19	4.6±0.17
Mahon Demias	31.9±0.78	13.1±0.17	11.3±0.27
Preto Amarelo	27.1±0.30	6.3±0.25	1.8±0.14
Emai19	31.3±0.22	12.7±0.14	3.5±0.16
Xiangmai25	30.6±0.26	12.7±0.23	7.3±0.25
Liangxing99	27.0±0.28	19.9±0.23	12.3±0.36
Zhengmai9023	36.4±0.61	25.9±0.39	14.0±0.32
Ningmai17	35.0±1.01	18.5±0.84	10.9±0.58
Xinong2000	27.7±1.33	12.5±0.37	5.2±0.22
Xinong223	34.7±0.43	13.0±0.29	7.3±0.29
Linyuan8	31.1±0.32	10.1±0.28	3.7±0.09
Yumai57	31.6±0.86	14.9±0.50	6.6±0.18
Huanong5	33.8±0.87	20.5±0.37	10.9±0.38
Huaimai16	33.6±1.40	13.4±0.19	5.7±0.35
Zhoumai16	30.5±1.08	12.3±0.27	7.4±0.18
Tainong292	39.1±0.57	22.6±0.35	13.7±0.22

Maximum quantum yield of PSII (F_v/F_m) varied between 0.805 ± 0.003 (Albidum24) and 0.785 ± 0.003 (Xinong223) under controlled conditions (Table 4.6). Chlorophyll fluorescence severely diminished among all the genotypes subjected to drought. Under 25% field capacity regime, *Fv/Fm* ratio varied between 0.680 ± 0.009 (Albidum24) and 0.417± 0.004 (Preto Amarelo). More severe drought stress (12% of the full field capacity) has further reduced *Fv/Fm* values ranged between 0.597 ± 0.007 (Albidum24) and 0.215 ± 0.006 (Xinong223).

Table 4.6 Genotypic variability in chlorophyll fluorescence (*Fv/Fm*) under controlled irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
Albidum24	0.805±0.003	0.680±0.009	0.597±0.007
Onohoiskaja4	0.801±0.005	0.585±0.008	0.369±0.009
Surhak Mestnyj	0.789±0.004	0.627±0.003	0.510±0.004
Pobeda	0.795±0.003	0.436±0.006	0.262±0.014
Kord Cl Plus	0.786±0.006	0.526±0.014	0.349±0.014
Mahon Demias	0.801±0.002	0.614±0.010	0.554±0.009
Preto Amarelo	0.787±0.003	0.417±0.004	0.231±0.007
Emai19	0.792±0.004	0.474±0.007	0.310±0.011
Xiangmai25	0.787±0.004	0.498±0.006	0.238±0.011
Liangxing99	0.790±0.004	0.624±0.003	0.532±0.008
Zhengmai9023	0.789±0.003	0.480±0.007	0.244±0.011
Ningmai17	0.801±0.003	0.514±0.004	0.307±0.011
Xinong2000	0.792±0.003	0.596±0.006	0.379±0.008
Xinong223	0.785±0.003	0.406±0.006	0.215±0.006
Linyuan8	0.793±0.003	0.615±0.004	0.417±0.007
Yumai57	0.791±0.003	0.457±0.007	0.329±0.005
Huanong5	0.794±0.003	0.647±0.009	0.464±0.014
Huaimai16	0.788±0.004	0.553±0.008	0.369±0.007
Zhoumai16	0.789±0.003	0.580±0.009	0.441±0.012
Tainong292	0.801±0.002	0.674±0.007	0.556±0.010

The highest stomatal conductance was found in Xiangmai25 variety (58.8 ± 0.88) followed by Surhak Mestnyj (57.8 ± 0.72) under irrigated conditions. The lowest stomatal conductance was observed in Yumai57 (35.1 ± 0.68) (Table 4.7). Under 25% field capacity irrigation regime, stomatal conductance ranged between 19.1 ± 0.36 and 6.5 ± 0.31, with variety Albidum24 having the highest Gs and Mahon Demias the lowest. Stomatal conductance was dramatically reduced under severe water stress, with Gs values ranging between 11.4 ± 0.49 and 0.4 ± 0.16. Genotype Kord Cl Plus had the highest stomatal conductance while Pobeda and Onohoiskaja4 showed the least values of Gs.

Table 4.7 Genotypic variability in stomatal conductance (Gs) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
Albidum24	51.0±0.57	19.1±0.36	7.0±0.29
Onohoiskaja4	44.2±0.48	8.1±0.32	0.4±0.18
Surhak Mestnyj	57.8±0.72	14.4±0.36	0.8±0.28
Pobeda	58.1±0.53	9.6±0.39	0.4±0.15
Kord Cl Plus	49.6±1.20	18.9±0.38	7.2±0.56
Mahon Demias	36.4±0.59	6.5±0.31	7.1±0.26
Preto Amarelo	35.9±0.80	9.5±0.30	0.5±0.15
Emai19	43.9±0.68	10.1±0.33	0.7±0.28
Xiangmai25	58.8±0.88	16.9±0.79	4.7±0.34
Liangxing99	56.1±0.58	14.8±0.73	0.7±0.27
Zhengmai9023	41.7±0.96	10.5±0.39	1.2±0.46
Ningmai17	50.0±0.37	16.1±0.52	2.5±0.61
Xinong2000	47.4±0.78	13.0±0.48	0.7±0.24
Xinong223	39.1±0.66	6.9±0.26	0.8±0.27
Linyuan8	50.4±0.42	6.9±0.38	0.5±0.21
Yumai57	35.1±0.68	8.8±0.41	0.6±0.30
Huanong5	36.9±1.04	11.3±0.54	1.3±0.37
Huaimai16	37.4±0.62	8.8±0.42	0.9±0.33
Zhoumai16	45.1±0.56	13.6±0.66	2.8±0.70
Tainong292	52.7±1.21	9.1 ±0.67	6.9±0.40

Under irrigated conditions, fresh shoot weight ranged between 1.50 ± 0.06g (Ningmai17) and 0.54 ± 0.06g (Kord Cl Plus) among the genotypes (Table 4.9). Plant shoot fresh weight varied between 0.74 ± 0.04g and 0.23 ± 0.03g under 25% field capacity irrigation with variety Zhengmai9023 having the highest Gs and Onohoiskaja4the lowest. Shoot fresh weight remarkably reduced under severe drought stress conditions as compared to control and mild (25% field capacity irrigation) stress. Shoot fresh weight varied between 0.37 ± 0.02g (Emai19) and 0.14 ± 0.01g (Kord Cl Plus).

Table 4.8 Genotypic variability in shoot fresh weight (FW) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
Albidum24	0.79±0.19	0.35±0.04	0.25±0.00
Onohoiskaja4	0.92±0.06	0.23±0.03	0.17±0.00
Surhak Mestnyj	1.09±0.29	0.44±0.13	0.21±0.01
Pobeda	0.73±0.08	0.31±0.05	0.16±0.01
Kord Cl Plus	0.54±0.06	0.29±0.01	0.14±0.01
Mahon Demias	0.77±0.15	0.43±0.04	0.25±0.02
Preto Amarelo	0.99±0.28	0.29±0.04	0.15±0.01
Emai19	0.85±0.30	0.63±0.20	0.37±0.02
Xiangmai25	0.84±0.20	0.34±0.04	0.19±0.01
Liangxing99	1.23±0.35	0.47±0.04	0.31±0.06
Zhengmai9023	1.09±0.18	0.74±0.04	0.16±0.01
Ningmai17	1.50±0.06	0.66±0.15	0.26±0.02
Xinong2000	0.76±0.09	0.43±0.09	0.24±0.02
Xinong223	0.73±0.02	0.36±0.07	0.24±0.03
Linyuan8	0.75±0.07	0.38±0.04	0.26±0.02
Yumai57	0.94±0.18	0.46±0.10	0.25±0.02
Huanong5	1.13±0.15	0.49±0.04	0.34±0.08
Huaimai16	0.85±0.13	0.37±0.04	0.29±0.04
Zhoumai16	0.89±0.05	0.35±0.03	0.25±0.01
Tainong292	0.97±0.17	0.49±0.05	0.34±0.02

The shoot dry weight showed a broad range of variability among genotypes under control conditions, ranging between 0.45 ± 0.27 and 0.09 ± 0.01, with Preto Amarelo having the highest and Kord Cl Plus the lowest SDW (Table 4.9). Under mild stress, shoot DW ranged between 0.20 ± 0.19g (Preto Amarelo) and 0.06 ± 0.02g (Kord Cl Plus). More severe drought stress (12% field capacity irrigation regime) significantly reduced shoot DW ranged between 0.14 ± 0.02g and 0.02 ± 0.01g (Table 4.9). Tainong292 produced highest biomass whereas Kord Cl Plus produced lowest.

Table 4.9 Genotypic variability in shoot dry weight (DW) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
Albidum24	0.15±0.02	0.11±0.01	0.08±0.01
Onohoiskaja4	0.14±0.01	0.10±0.00	0.06±0.01
Surhak Mestnyj	0.20±0.03	0.13±0.02	0.07±0.00
Pobeda	0.13±0.01	0.09±0.01	0.05±0.00
Kord Cl Plus	0.09±0.01	0.06±0.02	0.02±0.01
Mahon Demias	0.16±0.02	0.12±0.01	0.10±0.00
Preto Amarelo	0.45±0.27	0.20±0.19	0.06±0.00
Emai19	0.19±0.03	0.11±0.01	0.07±0.02
Xiangmai25	0.16±0.01	0.09±0.01	0.06±0.00
Liangxing99	0.22±0.04	0.14±0.01	0.09±0.01
Zhengmai9023	0.29±0.01	0.19±0.02	0.06±0.01
Ningmai17	0.20±0.00	0.14±0.01	0.10±0.00
Xinong2000	0.11±0.01	0.09±0.01	0.07±0.01
Xinong223	0.11±0.01	0.08±0.00	0.07±0.00
Linyuan8	0.12±0.01	0.08±0.01	0.07±0.00
Yumai57	0.12±0.01	0.09±0.01	0.08±0.01
Huanong5	0.17±0.01	0.14±0.01	0.10±0.02
Huaimai16	0.13±0.01	0.11±0.01	0.08±0.01
Zhoumai16	0.15±0.02	0.12±0.01	0.09±0.01
Tainong292	0.20±0.01	0.17±0.01	0.14±0.02

The relative water content in control plants was higher as compared to plants in drought stress and varied between 86.6% (Ningmai17) and 47.0% (Preto Amarelo) (Table 4.10). Relative water content was also adversely affected by drought stress in all barley genotypes. Relative water content varied between 80.4% (Emai19) and 55.8% (Onohoiskaja4) under mild stress (25% field capacity irrigation). Under severe drought stress, plant RWC was ranged between 81.2% and 41.1%, with Emai19 having the highest relative water content and Kord Cl Plus having the lowest.

Table 4.10 Genotypic variability in relative water content (RWC) of plants grown under control irrigation, 25% and 12% of full field capacity. Data are mean ± SEM (n = 6)

Genotypes	Control	25%FC	12%FC
Albidum24	79.8	67.2	67.0
Onohoiskaja4	84.7	55.8	47.8
Surhak Mestnyj	80.6	68.8	65.2
Pobeda	82.3	71.5	66.0
Kord Cl Plus	83.2	78.6	41.1
Mahon Demias	79.1	70.6	59.2
Preto Amarelo	58.7	76.9	47.0
Emai19	81.2	80.4	74.3
Xiangmai25	79.2	71.8	67.5
Liangxing99	81.5	70.2	71.6
Zhengmai9023	72.0	74.3	50.5
Ningmai17	86.6	75.9	61.3
Xinong2000	84.9	75.4	72.3
Xinong223	85.3	75.9	71.6
Linyuan8	83.6	76.8	73.4
Yumai57	86.1	77.6	66.4
Huanong5	84.6	71.8	70.6
Huaimai16	84.1	70.6	70.3
Zhoumai16	83.6	66.7	61.6
Tainong292	77.8	63.4	57.7

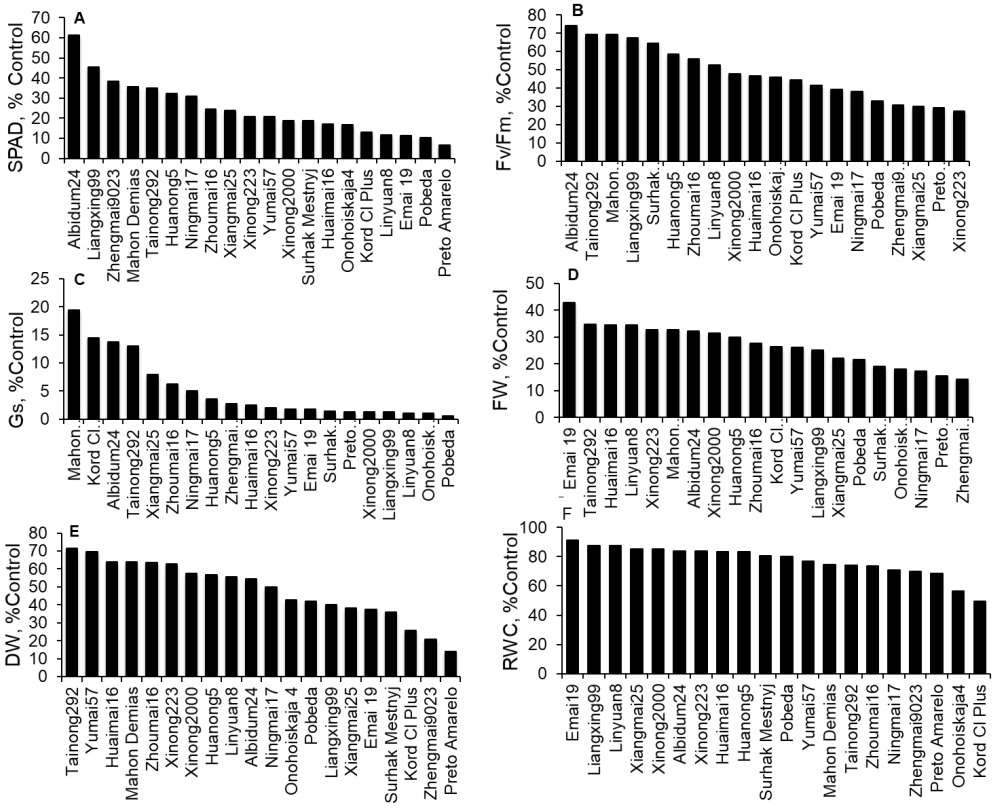

Figure 4.2 Relative chlorophyll content (SPAD)(A), chlorophyll fluorescence (*Fv/Fm*)(B), stomatal conductance (Gs)(C), fresh weight (FW)(D), dry weight (DW)(E) and relative water content (RWC)(F) of wheat genotypes grown under severe drought stress (12% of the full field capacity) shown as percentage of values under irrigated conditions

The relative changes in chlorophyll content (% control) differed among varieties (Fig. 4.2A) and ranged between 61.18% for Albidum24 and as little as 6.52 % for Preto Amarelo. The relative values of chlorophyll fluorescence of drought-stressed plants (% control) ranged between 75.5% for Albidum24 and 27.4% for Xinong223 (Fig 4.2B). On average, the stomatal conductance in drought stressed plants ranged between 19.37 % and 0.64% of the control, depending on the variety (Fig 4.2C). The relative fresh weight values of drought stressed plants (% control) varied between 42.56% and as low as 14.35 % with Emai19 having the highest value and Zhengmai9023 the lowest one (Fig 4.2D). For shoot dry weight, the relative values of stressed plants ranged between 71.16% (Tainong292) and 14.07% (Preto Amarelo) (Fig 4.2E). The relative water content in drought stressed plants ranged between 91% and 48%. The lowest reduction in RWC was found in Emai19 and the highest reduction was in Kord Cl Plus (Fig 4.2F).

4.2.3 Correlational Analysis

A significant positive correlation (R^2=0.22*,* significant at *P<0.05*) was found between SPAD values and shoot dry weight under irrigated conditions (Fig 4.3A). A strong positive correlation was seen between SPAD of plants grown under moderate (25% field capacity) and severe (12% field capacity) stress and dry biomass at severe stress (R^2=0.38 and R^2=0.37*;* significant at *P<0.01*) (Fig 4.3B & C).

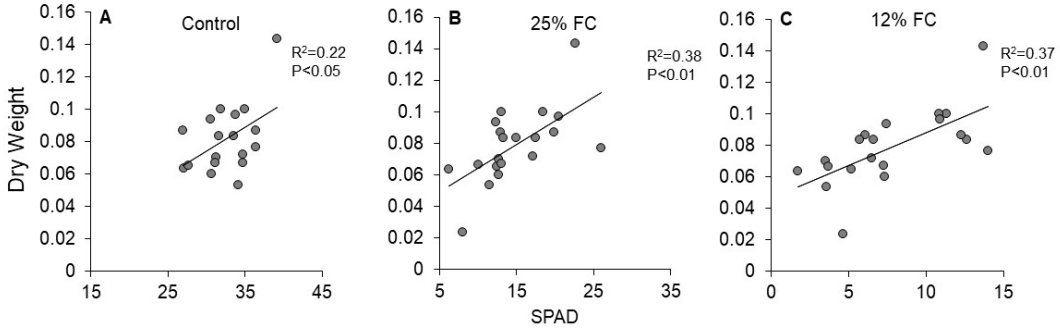

Figure 4.3 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and chlorophyll content (SPAD) of 20 wheat genotypes grown under control, 25% and 12% field capacity conditions

A strong positive correlation was found between shoot dry weight and *Fv/Fm* of plants grown under control, moderate (25% field capacity) and severe (12% field capacity) stress conditions (R^2=0.34; R^2=0.31; R^2=35: significant at *P<0.05*) (Fig 4.4).

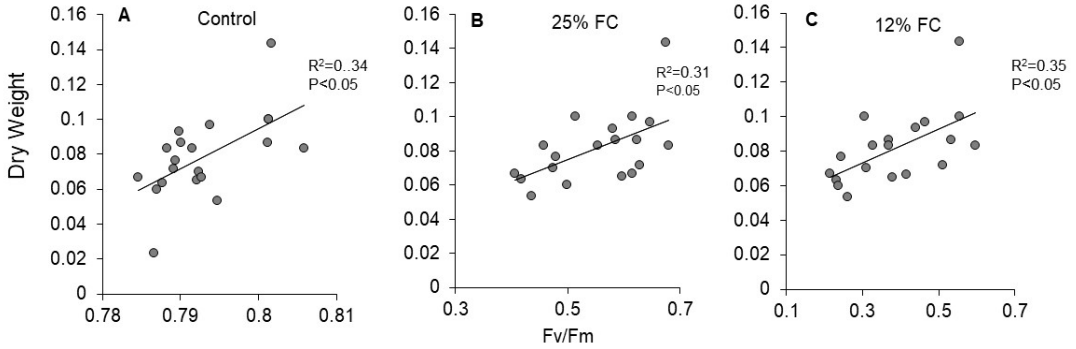

Figure 4.4 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and chlorophyll fluorescence (*Fv/Fm*) of 20 wheat genotypes grown under control, 25% and 12% field capacity conditions

There was no significant correlation between stomatal conductance (Gs) and shoot dry weight for plants grown under control and moderate (25% FC) stress conditions (Fig 4.5A&B). However, a strong positive correlation was observed between Gs of plants grown under severe (12% field capacity) stress conditions and the shoot dry weight (R^2=0.36; significant at *P<0.05*) (Fig 4.5C).

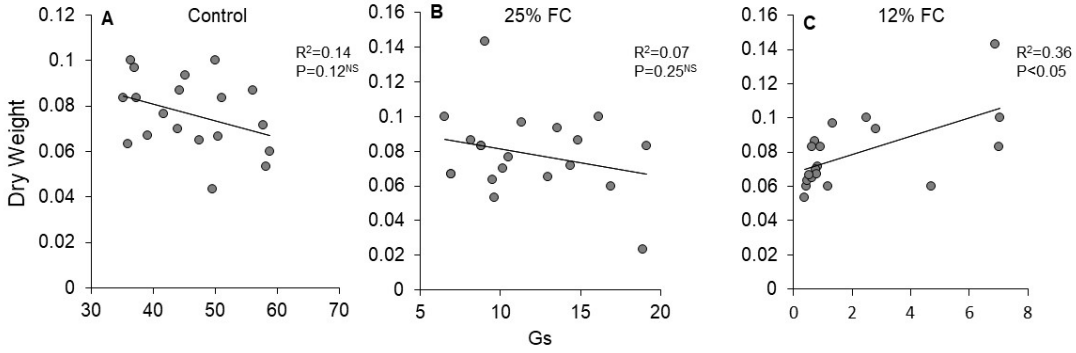

Figure 4.5 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and stomatal conductance (Gs) of 20 wheat genotypes grown under control, 25% and 12% field capacity irrigation conditions

A strong positive correlation was found between shoot fresh shoot weight and shoot dry weight for plants grown under control conditions (R^2=0.25, significant at *P<0.05*) (Fig 4.6A). However, there was no significant correlation between shoot fresh weight of plants grown under 25% drought stress and the shoot dry weight (Fig 4.6B). A strong correlation was observed between shoot fresh weight of plants grown under 12% field capacity and the shoot dry weight (R^2=0.47, significant at *P<0.01*) (Fig 4.6C).

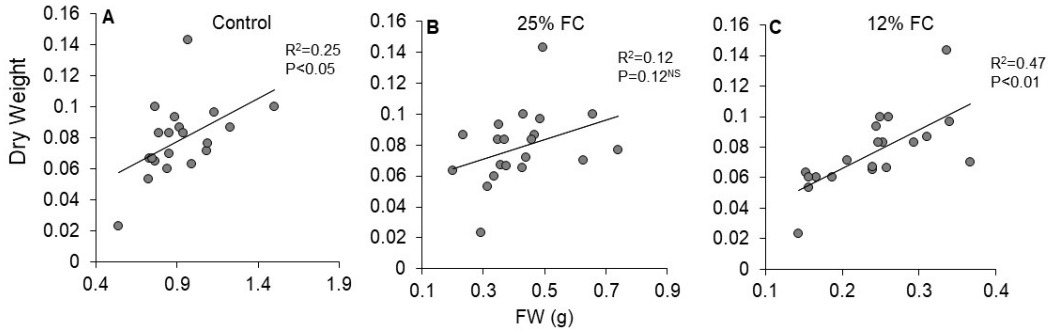

Figure 4.6 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and fresh weight (FW) of 20 wheat genotypes grown under control, 25% and 12% field capacity conditions

There was no significant correlation between relative water content (RWC) and shoot dry weight for plants grown under control conditions (Fig 4.7A). However, a positive and significant (R^2=0.22, significant at *P<0.05*) relationship was found between relative water content and dry shoot weight for plants grown under 25% field capacity irrigation (Fig 4.7B). No significant correlation was found between relative water content (RWC) and shoot dry weight for plants grown under 12% field capacity irrigation (Fig 4.7C).

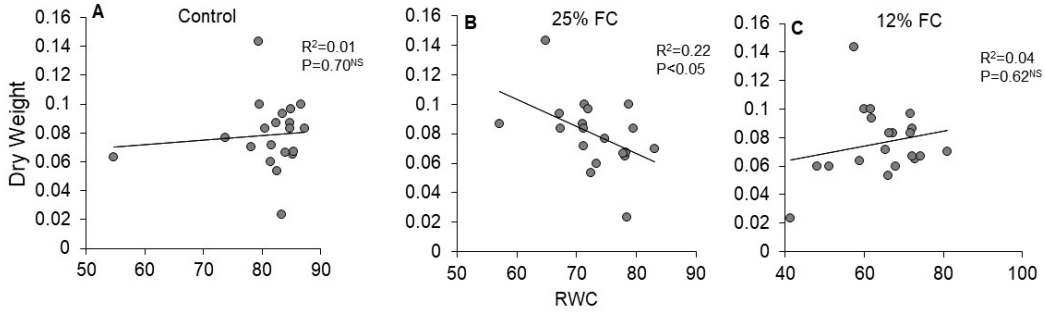

Figure 4.7 Correlation between dry weight of plants grown under severe drought stress (12% field capacity) and relative water content (RWC) of 20 wheat genotypes grown under control, 25% and 12% field capacity conditions

Table 4.11 The correlation matrix between shoot dry weight and major physiological characteristics of wheat plants grown under control and deficit irrigation conditions. SPAD - leaf chlorophyll content; Fv/Fm – maximum photochemical efficiency of PSII; Gs- stomatal conductance; FW - fresh weight; RWC - relative water content

	SPAD			**Fv/Fm**			**Gs**			**FW**			**RWC**		
	Control	25%FC	12%FC	Control	25%FC	12%FC	Control	25%FC	12%FC	Control	25%FC	12%FC	Control	25%FC	12%FC
DW	0.22*	0.38 **	0.37**	0.34*	0.31*	0.35*	0.14 NS	0.07NS	0.36*	0.25*	0.12NS	0.47**	0.01 NS	0.22*	0.04 NS

*=P<0.05
**= P<0.01
***= P<0.0001
NS= Non-significant

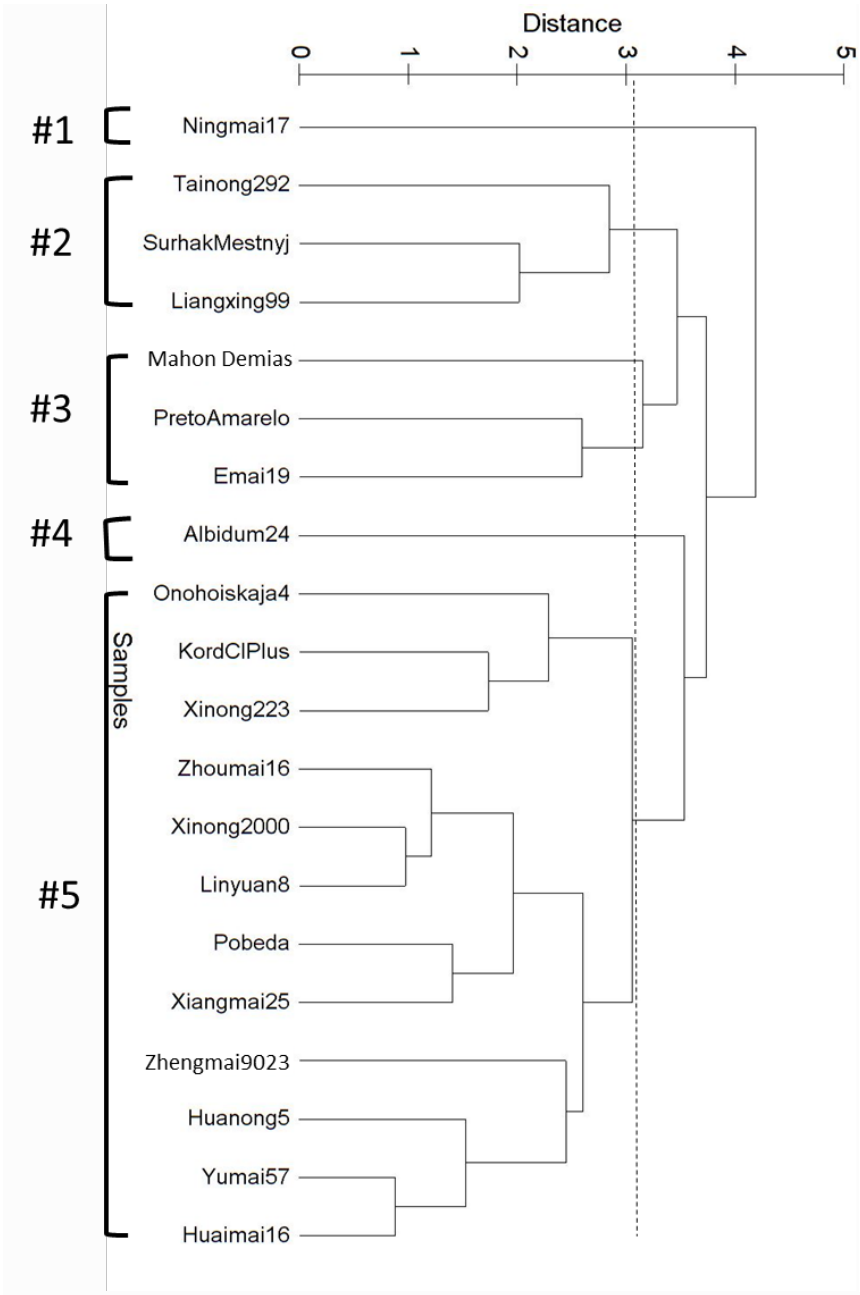

Figure 4.8 The hierarchical cluster analysis of 20 wheat genotypes grown under control conditions. Plants are grouped into 4 groups based on chlorophyll content (SPAD), chlorophyll fluorescence (Fv/Fm), stomatal conductance (Gs), fresh weight (FW), dry weight (DW) and relative water content (RWC). The dendrogram shows fusion levels at which the groups join. The vertical dashed line represents the truncation into four genotype groups using Ward's agglomerative clustering algorithm

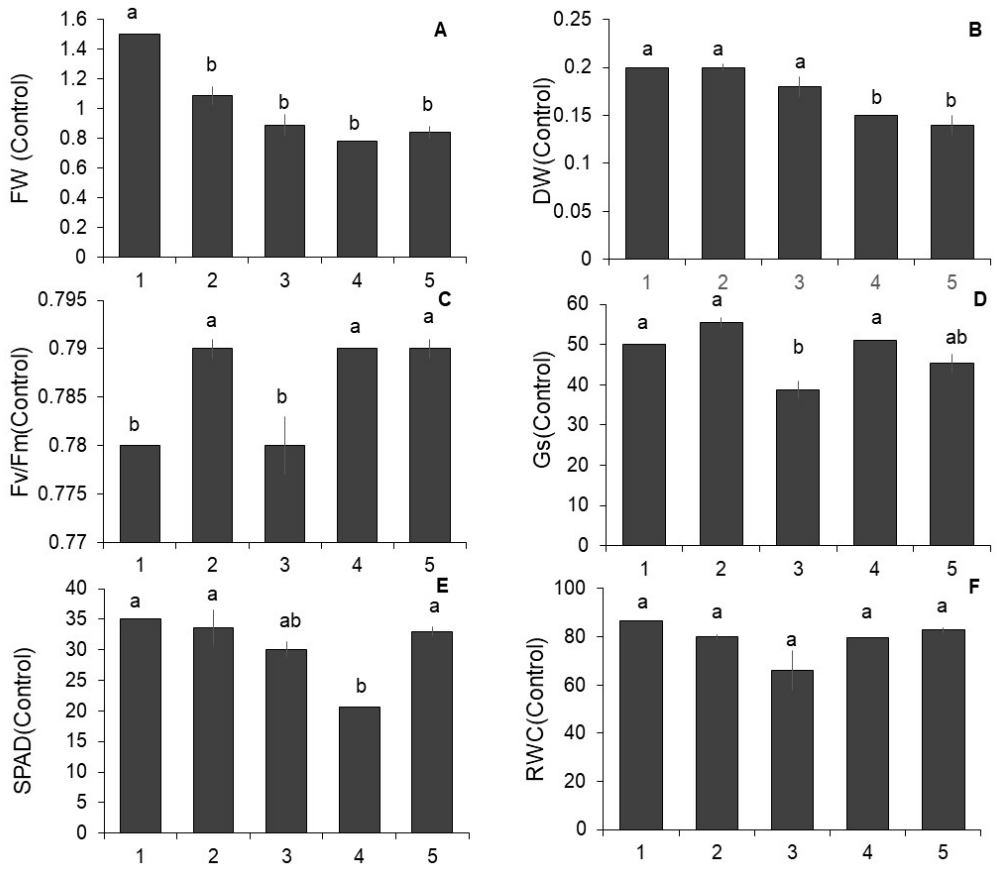

Figure 4.9 Comparison of the groups produced by cluster analysis, showing the differences between groups in A) fresh weight, B) dry weight, C) chlorophyll fluorescence, D) stomatal conductance, E) SPAD and F) relative water content, respectively, for control condition. Different lowercase letters indicate the significance difference between clusters at $P<0.01$

4.2.4 Cluster analysis for plant grown at full field capacity water content

Cluster analysis based on agronomical and physiological characteristics divided twenty wheat genotypes grown under control conditions into five clusters (Fig 4.8). Mean values for each cluster are plotted in Fig 4.9. Cluster 1 have only Ningmai17 genotype. This genotype had highest fresh weight, chlorophyll content, relative water content and lowest Fv/Fm (chlorophyll fluorescence) under irrigated conditions (Fig 4.9). Cluster 2 comprised of Tainong292, Surhak Mestnyj and Liangxing99. These genotypes had highest Fv/Fm, dry weight and stomatal conductance. Cluster 3 consisted of Mahon Demais, Preto Amarelo, and Emai 19. These genotypes had lowest Fv/Fm, stomatal conductance and relative water content. Cluster 4 have only Albidum24. This genotype had least fresh weight and chlorophyll content. Cluster 5 consisted of twelve genotypes (Onohoiskaja4, Kord Cl Plus, Xinong223, Zhoumai16, Xinong2000, Linyuan8, Pobeda, Xiangmai25, Zhengmai9023, Huanong5, Yumai57 and Huaimai16) and these genotypes had least dry weight (Fig 4.9).

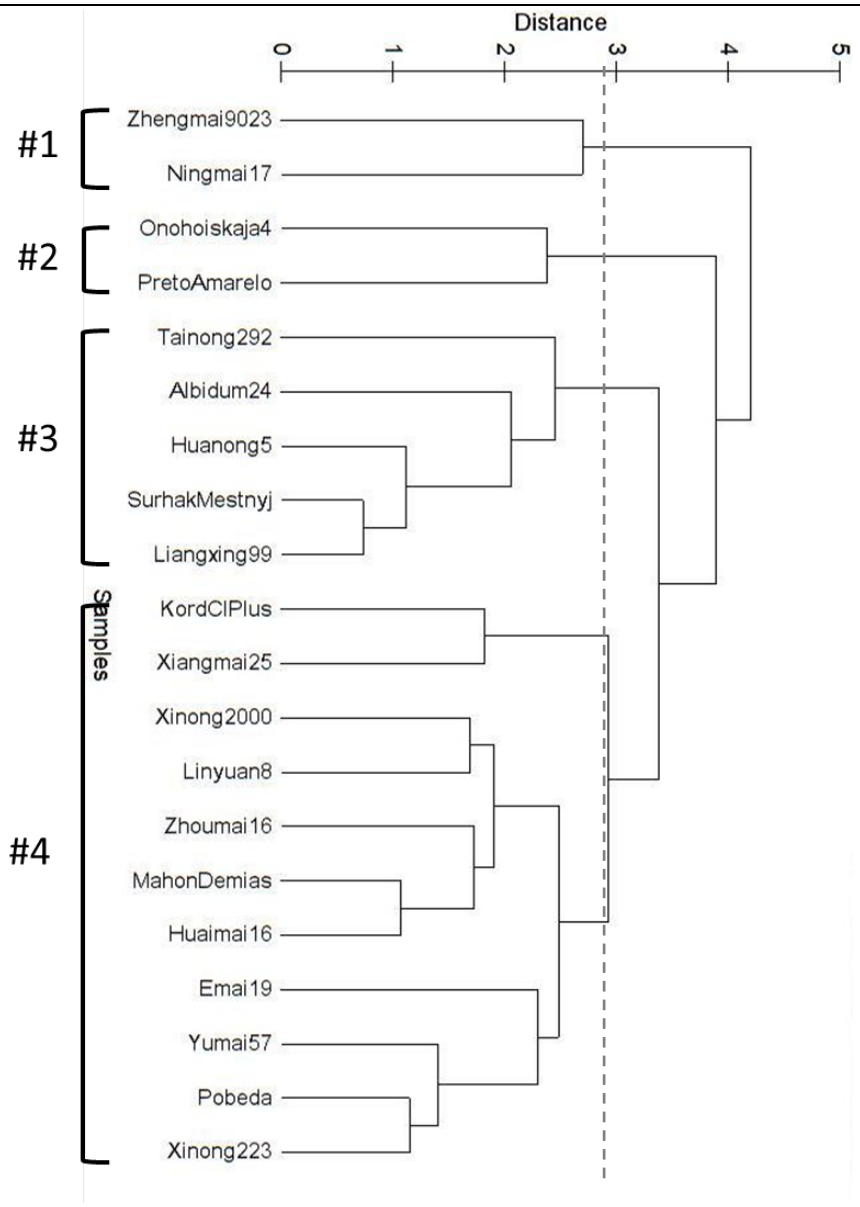

Figure 4.10 The hierarchical cluster analysis of 20 wheat genotypes into 4 groups grown under moderate (25% field capacity) drought stress conditions. Plants are grouped based on chlorophyll content (SPAD), chlorophyll fluorescence (*Fv/Fm*), stomatal conductance (Gs), fresh weight (FW), dry weight (DW) and relative water content (RWC). The dendrogram shows fusion levels at which the groups join. The vertical dashed line represents the truncation into four genotype groups using Ward's agglomerative clustering algorithm

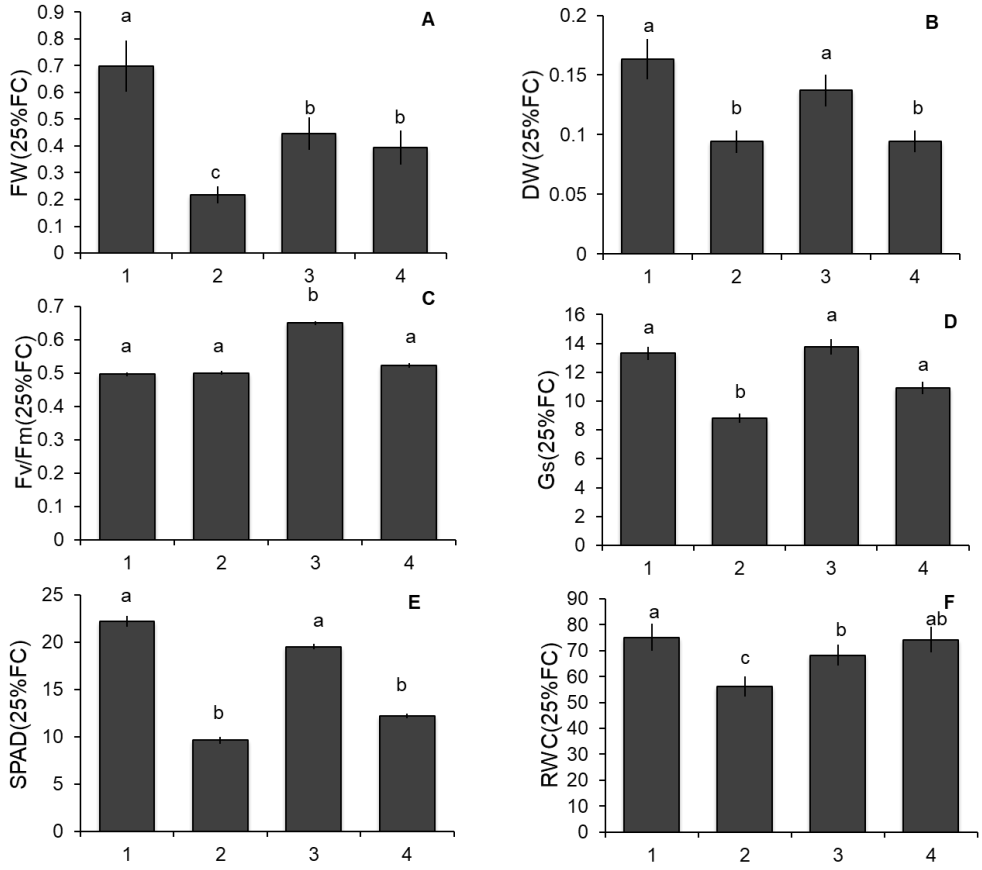

Figure 4.11 Comparison of the groups produced by cluster analysis in Fig 4.10 for plants grown under moderate (25% field capacity) stress conditions, showing the differences between groups in fresh weight (A), dry weight (B), *Fv/Fm* (C), stomatal conductance (D), SPAD (E), relative water content (F). Different lowercase letters indicate the significance difference between clusters at $P<0.01$

4.2.5 Cluster analysis for plants grown at 25% field capacity water content

Cluster analysis based on agronomical and physiological traits divided twenty wheat genotypes grown under moderate (25% field capacity) conditions into four clusters (Fig 4.10). Cluster 1 comprised of Zhengmai9023, Ningmai17. These genotypes had highest fresh weight, dry weight, chlorophyll content, relative water content (Fig 4.11). Cluster 2 consisted of Onohoiskaja4 and Preto Amarelo and these genotypes had least Gs and relative water content, dry weight, fresh weight, chlorophyll content. This cluster can be referred as the most sensitive cluster among all clusters under moderate stress conditions. Cluster 3 was comprised of five genotypes (Tainong292, Albidum24, Huanong5, Surhak Mestnyj, and Lianxing99). These genotypes had highest stomatal conductance and Fv/Fm. Cluster 4 contained eleven genotypes (Kord Cl Plus, Xiangmai25, Xinong2000, Linyuan8, Zhoumai16, Mahon Demais, Huaimai16,

Emai19, Yumai57, Pobeda and Xinong223). These genotypes had intermediate values for all the parameters (Fig 4.11).

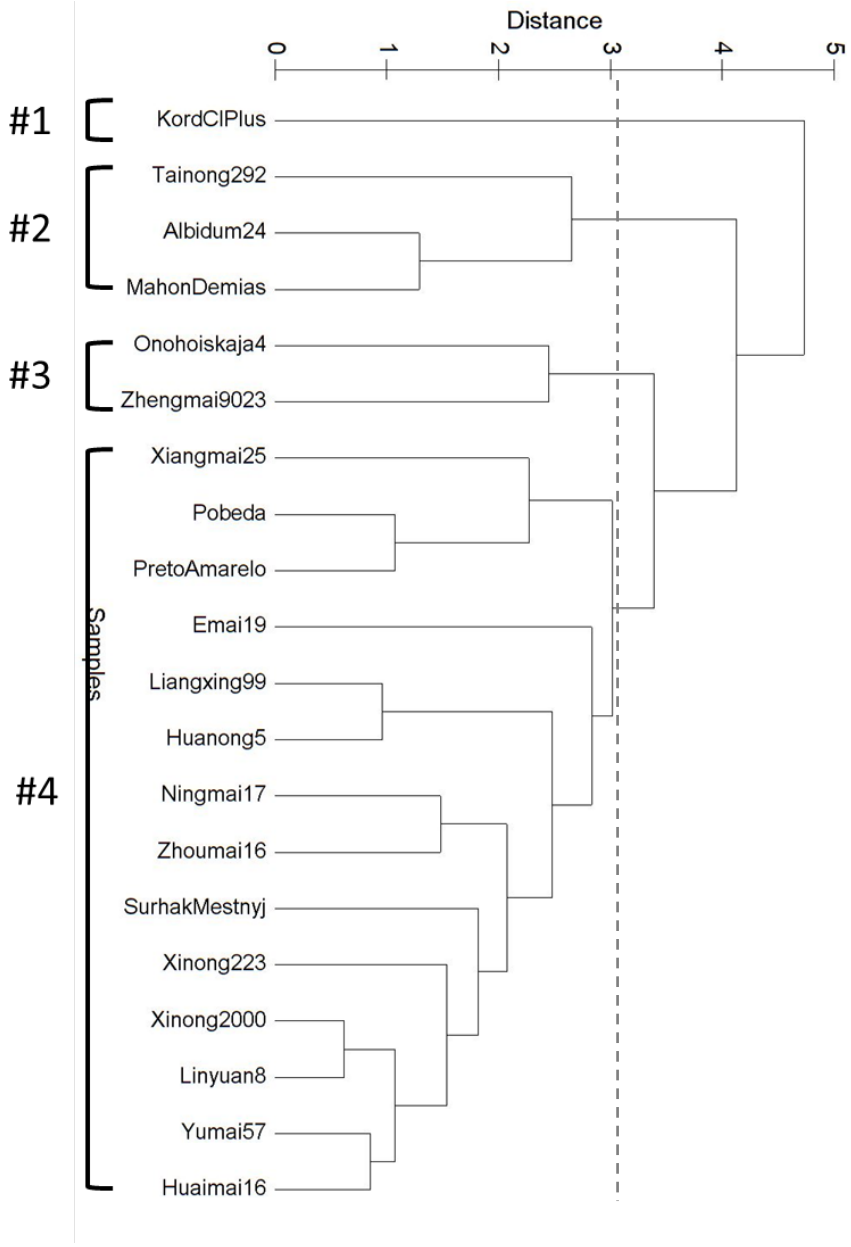

Figure 4.12 The hierarchical cluster analysis of 20 wheat genotypes into 4 groups grown under severe (12% field capacity) drought stress conditions. Plants are grouped based on chlorophyll content (SPAD), chlorophyll fluorescence (*Fv/Fm*), stomatal conductance (Gs), fresh weight (FW), dry weight (DW) and relative water content (RWC). The dendrogram shows fusion levels at which the groups join. The vertical dashed line represents the truncation into four genotype groups using Ward's agglomerative clustering algorithm.

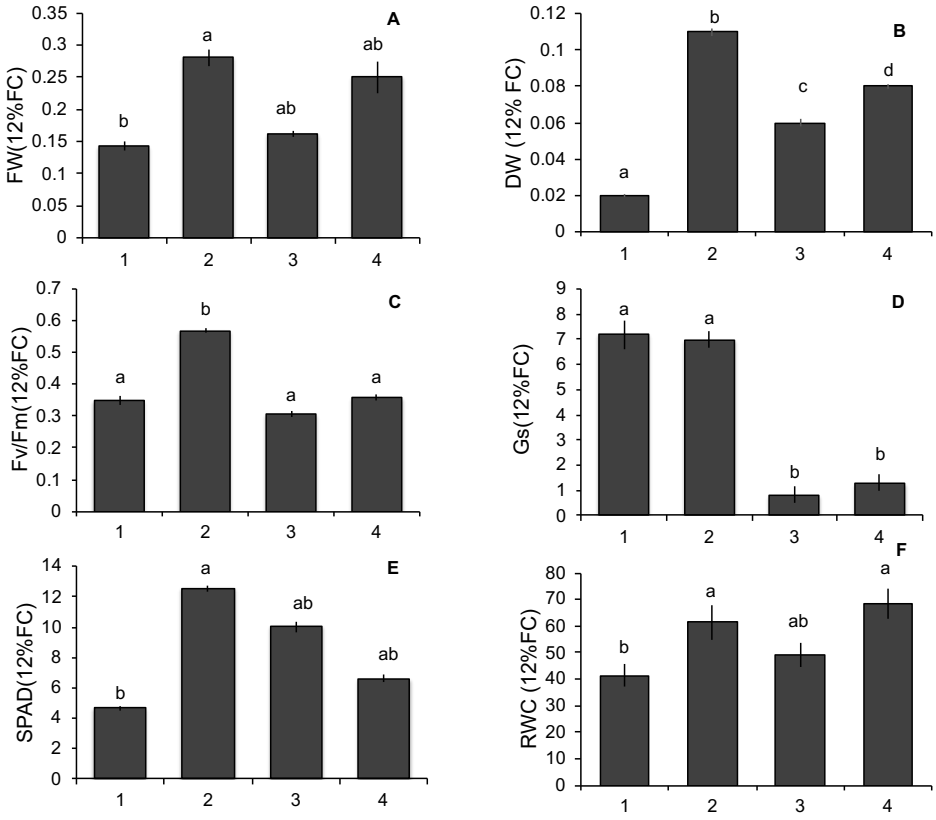

Figure 4.13 Comparison of the groups produced by cluster analysis in Fig 4.12 for plants grown under severe (12% field capacity) stress conditions, showing the differences between groups in fresh weight (A), dry weight (B), *Fv/Fm* (C), stomatal conductance (D), SPAD (E), relative water content (F), respectively. Different lowercase letters indicate the significance difference between clusters at $P<0.01$

4.2.6 Cluster analysis for plants grown at 12% field capacity water content

Cluster analysis based on physiological and agronomical traits divided twenty wheat genotypes grown under severe stress (12% field capacity) conditions into four clusters (Fig 4.12). The first group contained only one genotype (Kord Cl Plus). This genotype had lowest chlorophyll content, fresh weight, dry weight, relative water content, moderate Fv/Fm and highest stomatal conductance (Fig 4.13). Thus, Cluster 1 can be referred as a sensitive group. Cluster 2 had three genotypes (Tainong292, Albidum24, Mahon Demais). These genotypes had highest fresh weight, dry weight, chlorophyll content, Fv/Fm and moderate stomatal conductance and relative water content. As a result, Cluster 2 can be referred as the most tolerant group amongst all. Cluster 3 had only two genotypes (Onohoiskaja4, Zhengmai9023). These genotypes had lowest Gs and Fv/Fm values and had moderate values for all other parameters. Cluster 3 can also be referred moderate sensitive group (Fig 4.13). Cluster 4 gathered together all the

fourteen genotypes (Xiangmai25, Pobeda, Preto Amarelo, Emai19, Liangxing99, Huanong5, Ningmai17, Zhoumai16, Surhak Mestnyj, Xinong223, Xinong2000, Linyuan8, Yumai57 and Huimai16). These genotypes had highest relative water content and moderate values for stomatal conductance, fresh weight, dry weight, chlorophyll content and chlorophyll fluorescence and can be referred as a moderately tolerant group.

4.2.7 Principle Component Analysis

The relationships between the different variables and genotypes with respective principal components are further illustrated by the principal component biplot in illustrated in Fig 4.15 for 12% field capacity irrigation conditions. The first two components explained 69.93% of the total variation between parameters. Smaller angles between dimension vectors in the same direction indicated high correlation of the variable traits in terms of discriminating genotypes. The principle component 1 (PC1) explained 46.49% of the variation and showed positive correlation with stomatal conductance, SPAD, dry weight, fresh weight and Fv/Fm negative correlation with relative water content. Hence, the first dimension can be referred as the best indicator of drought tolerance. The genotypes with higher values of PC1 are expected to be drought tolerant. The principle component 2 (PC2) describes 23.44% of total variability. The genotypes with lowest scores for PC1 and PC2 are drought sensitive genotypes.

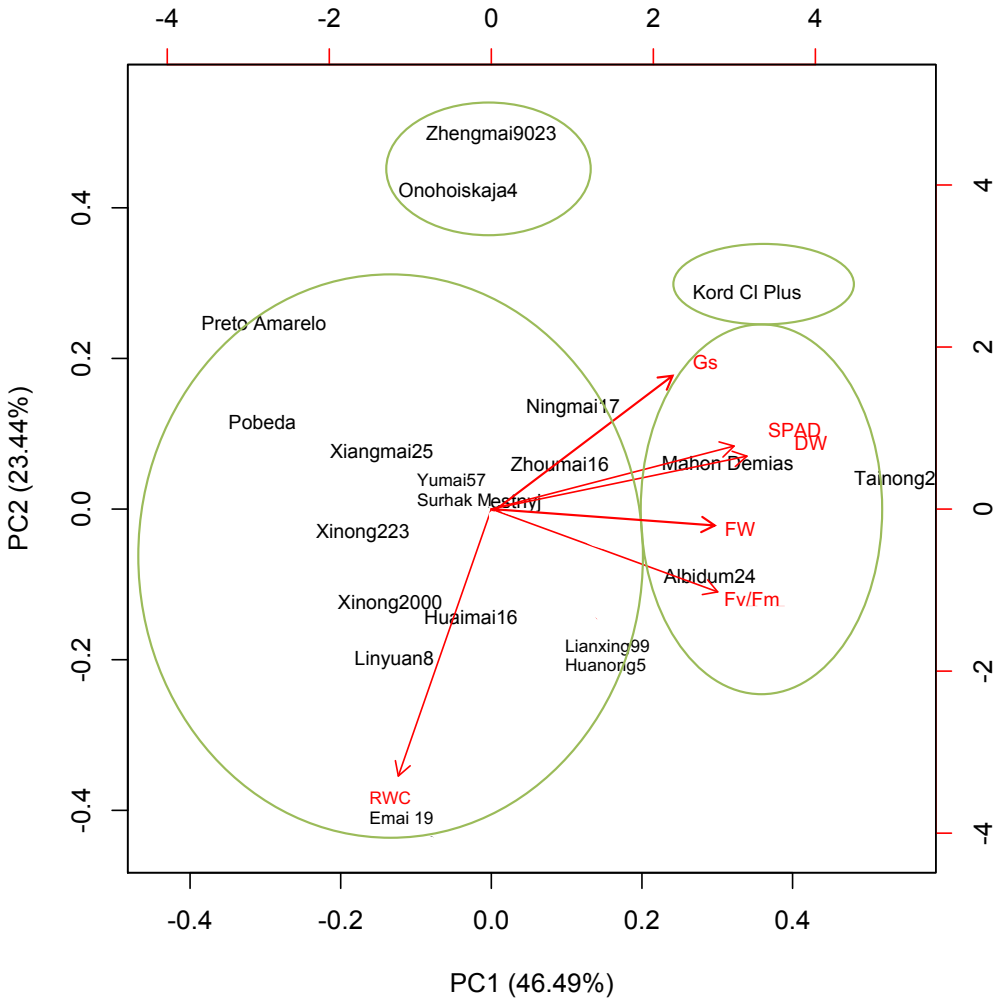

Figure 4.14 Biplot for drought tolerance indices in 20 wheat genotypes based on first two components measured under 12% field capacity. DW: Dry weight; Gs: Stomatal conductance; Fv/Fm: Chlorophyll fluorescence; RWC: Relative water content; FW: Fresh weight; SPAD: Chlorophyll content.

4.3 Discussion

4.3.1 Genotypic variations in wheat under drought stress

Development of drought tolerant wheat genotypes is the prime goal of wheat breeders. Effective germplasm screening for drought tolerance particularly under managed drought conditions is an efficient way of selecting materials for advanced breeding programs. Twenty wheat genotypes assembled from different origins and habitats were used in this study (Table 2.2 in Materials and Methods). Significant genotypic differences were observed among all the genotypes under drought stress. The first

experiment was performed by visual evaluation of plants responses to drought based on drought damage index (DDI). Complete withholding of water for seven weeks induced variable symptoms of leaf senescence (chlorosis and necrosis) in all genotypes. Leaf chlorosis and necrosis are important visible symptoms associated with drought stress. These symptoms generally develop as a result of chlorophyll degradation and with the deficiency of essential nutrients under drought stress (Forde, 2000; Hörtensteiner and Kräutler, 2011; Pessarakli et al., 2015). Out of twenty genotypes, five (Mahon Demias, Albidum24, Yumai57, Huiamai16 and Tainong292) were classified as highly drought tolerant (with DDI <6.5). These genotypes also accumulated relatively high shoot biomass and showed improved physiological characteristics under severe water deficit regime (12% field capacity water content). The fourth group was comprised of Liangxing99, Surhak Mestnyj, Pobeda and Zhenmgmai9023 genotypes exhibiting highest drought damage index (DDI over 9) and was termed as drought susceptible. The variation in drought tolerance in twenty genotypes could be explained by their origin. The highly tolerant genotypes Tainong292, Humai16 and Mahon Demias were originated from hot dry regions of China (Shandong, Jiangsu) and Spain (Fig 4.15). On the contrary, the sensitive genotypes Onohoiskaja4 and Kord Cl Plus were originated from high rainfall areas of Russia and Australia and extremely low drought exploited areas of China (Zhengmai9023). Previously Peleg et al. (2005) found a strong association between the origin of hot dry climate and drought tolerance of wild emmer wheat population. The sensitive genotypes somehow showed different trend in both experiments. Based on lowest accumulation of biomass and values of other studied physiological traits, Kord Cl Plus, Onohoiskaja4 and Zhengmai902 were highly sensitive genotypes whereas Pobeda, Lianxing99 and Surhak Mestnyj were in the moderately tolerant group (sensitive in the previous experiment). As leaf senescence is directly associated with chlorophyll content in plants, therefore the high damage index in Pobeda, Lianxing99 and Surhak Mestnyj can be possibly explained by the low chlorophyll content in moderately tolerant group containing these genotypes (Fig 4.13). The results of the current study suggested that the germplasm pool used in this study could be a rich source of genetic diversity for breeding purposes as indicated by differential genotypic responses to drought stress.

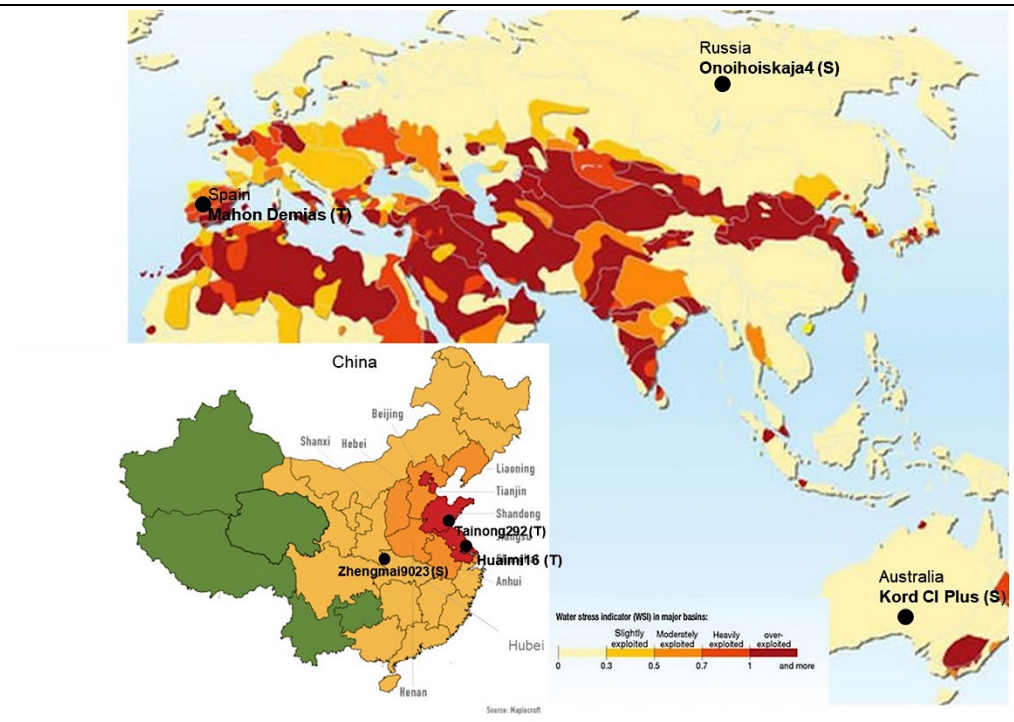

Figure 4.15 Geographical origin of highly drought tolerant and sensitive genotypes. T-drought tolerant genotypes, S -drought sensitive.

4.3.2 Comparison between wheat and barley:

Barley and wheat are amongst most important cereal crops. Compared to wheat, barley is generally considered to be more tolerant in context to high yield potential under drought stress (Woldeamlak et al., 2006). To pinpoint the physiological mechanisms underlying this greater drought tolerance in barley, I have evaluated the differences between physiological responses to drought in different wheat and barley genotypes (data from Chapter 4). For the genotypes (20 wheat and 30 barley genotypes) considered, there was no difference in the range of drought tolerance found in wheat and barley genotypes, indicating a similar range in drought tolerance and sensitivity as clearly shown in Fig 4.16 for the relative SPAD values (58% - 4% and 61% - 6% for barley and wheat respectively), as SPAD found to be a good a parameter to estimate drought tolerance in current chapter and chapter 3. Nevertheless, the data indicates that in these two cereals contrasting mechanisms act in coordination to determine tolerance or sensitivity at the whole plant level. Indeed, the relative stomatal conductance (Gs) and relative water content (RWC) compared to control conditions was found to be high for barley (Gs=23% - 0.7%, RWC=99% - 55%) as compared to wheat (Gs=19% - 0.6%, RWC=91% - 48%) (Fig 4.16). RWC can be maintained by either closing the stomata

or osmotic adjustment (Aroca, 2012). This suggests that tolerance in wheat may be achieved by closing the stomata, while in barley drought tolerance is likely to be achieved via osmotic adjustment, which then enables greater stomatal opening compared to wheat under drought conditions. Osmotic adjustment (OA) is the main factor in regulating cell turgor and plant water content and allows stomata to stay partially open by maintaining CO_2 fixation under severe drought stress (Ahmad et al., 2018). OA involves the accumulation of compatible solutes and inorganic ions in response to water stress. Consequently, the cell osmotic potential decreases which attracts the water into the cells and enables turgor to be maintained (Blum et al., 1996).

These findings could have some important implications for the final yield potential of the plants. Here only the vegetative stage was considered, and this could explain the similar range of drought tolerance found in wheat and barley. However, this greater osmotic adjustment and stomatal opening in barley compared to wheat, in the long-term has the potential to increase grain yield. Indeed, in the long-term, stomatal closure results in the inhibition of photosynthesis due to decrease in internal CO_2 concentration (Akıncı and Lösel, 2012). On the other hand, plants that can adjust osmotically can sustain high photosynthetic rate because of more favourable water status and high stomatal conductance, which in return, results in higher crop productivity (Allahverdiyev, 2015; Cannella et al., 2016)

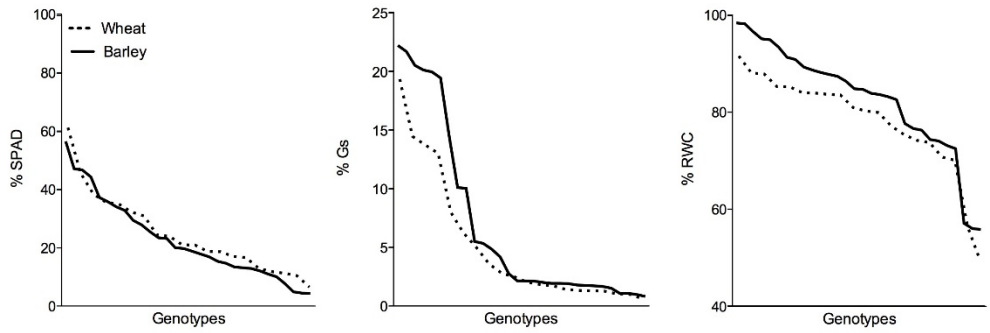

Figure 4.16 Relative SPAD (chlorophyll content), Gs (stomatal conductance), RWC (relative water content) of wheat and barley genotypes grown under severe drought stress (12% of full field capacity) shown as percentage of values under irrigated conditions.

4.3.3 Cluster analysis

Cluster analysis for plants growing under severe drought stress (12% field capacity irrigation) revealed that Cluster 2 comprised of Tainong292, Mahon Demias and Albidum24 was referred as the highly drought tolerant (Fig 4.13). These genotypes maintained relatively high shoot fresh weight, shoot dry weight, chlorophyll content and chlorophyll fluorescence under severe drought (12% of full field capacity). Biomass has strong relationship with the chlorophyll (see PCA plot where SPAD is grouped with the shoot dry weight). These genotypes maintained high relative water content. High RWC could be maintained due to the accumulation of solutes as a consequence of osmoregulation to maintain cell turgor under water stress as proposed by Larkunthod et al. (2018). Cluster 4 (Xiangmai25, Pobeda, Preto Amarelo, Emai19, Liangxing99, Huanong5, Ningmai17, Zhoumai16, Surhak Mestnyj, Xinong223, Xinong2000, Linyuan8, Yumai57 and Huimai16) is referred as moderately tolerant. Cluster 4 exhibited highest relative water content as compared with other clusters. Maintaining high relative water content is an important plant adaptive response to water stress (Keyvan, 2010). Plants generally maintained high plant water due to reduced transpiration rate by closing their stomata as expressed by low Gs values (Fig 4.13) (Hossain et al., 2015; Yan et al., 2016). Cluster 1 comprised of only one genotype (Kord Cl Plus) and exhibited high susceptibility to drought stress. This genotype had lowest chlorophyll content, chlorophyll fluorescence (*Fv/Fm*) and biomass under severe drought stress. Several other studies have shown that the reduction in chlorophyll content and damage to photosystem II was more severe to sensitive genotypes (Li et al., 2006; Rahbarian et al., 2011). The decrease in chlorophyll content under drought stress could be mainly due to chloroplasts damage caused by reactive oxygen species leading to reduced photosynthesis which ultimately decrease the biomass production (Del Pozo et al., 2016; Lopes and Reynolds, 2012; Rivero et al., 2007). This genotype (Kord Cl Plus) also had least relative water content and highest Gs under severe stress. High Gs increases the transpiration rate and therefore plants had reduced water content (Ewers, 2013). Cluster 3 (Onohoiskaja4 and Zhengmai9023) is also classified as drought sensitive on the basis of low shoot fresh weight, shoot dry weight, stomatal conductance and Fv/Fm ratio. The reduction in biomass in these genotypes could be related to disruption of photosynthesis by low stomatal conductance and damage to PSII reaction centre (Paknejad et al., 2007).

Chapter 5. Revealing key physiological traits conferring drought stress tolerance in barley

5.1 Introduction

Drought represents the most devastating abiotic stress factor limiting plant yield and productivity and global climate changes are likely to further limit water availability for plant growth. When plants are confronted with water stress, they respond to the stress condition by a broad array of morphological, physiological and biochemical adaptive mechanisms (Fang and Xiong, 2015). At the initial stages of drought stress, plants usually adapt to drought by inducing some morphological responses such as escaping from drought by shortening their life cycle before the onset of dry season, developing an efficient root system for extracting water and nutrients from deep layers, partial closing their stomata to reduce transpiration, reducing leaf area, leaf rolling, developing glaucousness on leaves, and transforming plant metabolism to match with the available carbon resource (Farooq et al., 2009b; Hu and Xiong, 2014; Song et al., 2016). With the severity of stress, plants undergo some important physiological changes to cope with drought stress such as accumulation of compatible organic solutes and increase in inorganic ion concentrations that contribute in osmotic adjustment, detoxification of reactive oxygen species and membrane stabilization (Ashraf et al., 2011; Hussain Wani et al., 2013).

Barley is the fourth most important cereal crop worldwide after wheat, corn, and rice. Barley predominantly considered as a food crop in many developing countries, where it is often subjected to extreme drought stress during their life cycle that significantly limits production (Nazari and Pakniyat, 2010). Root length is associated with plant water uptake and nutrient concentrations in the plant, therefore it could be used as most effective trait measuring drought tolerance, and some reports suggest that barley develops an extensive root system to cope with drought stress and produced more yield during its vegetative growth (Chloupek et al., 2010; El-Denary and El-Shawy, 2016). However, higher root length comes with the higher carbon cost, so the essentiality of this trait for maintaining crop productivity under stress conditions remains to be validated.

Control of stomata aperture is essential to optimise the rate of transpiration under changed conditions. As such, stomatal conductance can be used as a potential indicator of drought tolerance. Previous attempts to correlate drought tolerance in barley with stomatal conductance have found positive correlation between Gs and grain yield under drought conditions (Behbahanizadeh et al., 2014); the same trend has been seen in some other species (Bahar et al., 2009; El-Sabagh et al., 2017). Under water stress, plants close their stomata to prevent water loss, consequently decrease photosynthesis via reduced entry of CO_2 (Pinheiro and Chaves, 2010). However, to cope with water limited conditions, stomata can adjust their aperture by opening to optimize CO_2 influx and close to minimize transpiration rates (Ainsworth and Rogers, 2007). Therefore, modifications in Gs in response to water stress can be attributed to changes in stomatal density or altered stomatal aperture. It is suggested by various studies that reducing stomatal density may improve water use efficiency and drought tolerance in plants (Hughes et al., 2017; Xu and Zhou, 2008). There are still few studies showing that reduced soil moisture content continually generated new stomata and hence resulted in significant increase in stomatal density (Zhao et al., 2015). However, there is an ambiguity that whether or not stomatal density has a significant relation to plant drought tolerance.

Drought-induced osmotic stress requires osmotic adjustment, which can be achieved by either increased uptake of inorganic ions (mainly K^+, Na^+ and Cl^-) or increase accumulation of compatible solutes (Chen and Jiang, 2010). Accumulation of compatible solutes not only plays a leading role in cell turgor maintenance but also in protection of different cell structures (Anjum et al., 2017). Various studies indicated that both inorganic and organic osmolytes contribute to drought tolerance; their relative contribution, however, is still disputed in literature. It was argued that organic osmolytes are present at low concentrations in the cytosol, and their synthesis comes with significant energy cost (Shabala and Shabala, 2011). This notion is echoed by the recent study on barley reporting the absence of any significant correlation between drought tolerance and organic osmolytes content (Dbira et al., 2018). The authors concluded that accumulation of these solutes was a general conserved response and not suitable as a beneficial indicator of drought tolerance in these species. So, should higher *de novo* synthesis of compatible solutes for the osmotic adjustment be targeted in barley breeding programs?

Inorganic ions make significant contribution towards osmotic adjustment (Chen and Jiang, 2010). Three major candidates to be considered are K^+, Na^+ and Cl^-. Of these, potassium (K^+) is consistently reported as a major solute involved in OA in different crops (Damon et al., 2011; Ogawa and Yamauchi, 2006), contributing up to 78% of osmotic adjustment (Morgan, 1992). The osmotic adjustment through K^+ uptake is considered more energy efficient in plants suffering water deficit conditions (Bergmann, 2016). Therefore, the accumulation of K may be more important than the production of organic osmolytes during the initial adjustment phase.

However, passive K^+ uptake under drought conditions is highly unlikely from the thermodynamic point of view. Can plants use Na^+ as "cheap" inorganic osmolyte? While it may come with the energetic benefits, accumulation of high Na may cause metabolic disturbances (Maathuis, 2013). In hydrated form, Na and K are structurally very similar therefore Na can play the role of K to maintain ionic balance, regulating osmotic potential and contributing to vacuolar functions (Krishnasamy et al., 2014; Mäser et al., 2002; Subbarao et al., 2003). In addition of K and Na, the contribution of chloride in osmotic adjustment may be rather substantial (Shabala and Shabala, 2011). Several studies suggested that osmotic adjustment in roots and leaves are predominantly due to chloride accumulation as compared to other ions (Franco-Navarro et al., 2015; Silva et al., 2018). However, other reports showed that chloride did not contribute significantly towards osmotic adjustment under drought stress (Ma et al., 2004). Is this the case for barley? Which inorganic ion makes a biggest contribution towards osmotic adjustment?

The aim of the study was to fill in the above gaps in our knowledge and advance our understandings of the mechanisms underlying drought stress tolerance in barley. This was achieved by comparing anatomical and physiological traits of contrasting barley genotypes and linking the overall drought tolerance of these barley genotypes with changes in plant water related traits, stomatal characteristics and the contribution of organic and inorganic osmolytes towards the root and shoot osmotic adjustment.

5.2 Results

Based on the drought damage index and cluster analysis of the previous experiments, seven genotypes contrasting in their drought tolerance ability were selected. These genotypes were clustered into three groups: tolerant (drought damage index ≤ 6.5), moderate (DDI = 8.25 to 8.75) and sensitive (DDI = 9). There are two varieties in tolerant group, Numar and ZUG293. The moderate group included Commander, Fleet, and X123, while sensitive group contained Franklin and Gairdner. Whole plant traits including anatomical and physiological characteristics were measured in response to three different water regimes (control; and controlled drought – deficit irrigation at 25% and 12% of full field capacity, respectively).

5.2.1 Root length (RL)

A significant difference was found between all the genotypes under drought stress. Under 25% field capacity, root length of tolerant group significantly increased up to 59%, while in moderate and sensitive genotypes relative root length decreased by 7% and 47%, respectively (Fig 5.1D). Under 12% field capacity irrigation, tolerant group exhibited an increase of 26%, while moderate and sensitive genotypes showed a reduction in relative root length by 19% to 39%, respectively (Fig 5.1E). The results obtained from the correlational analysis revealed the existence of the positive but non-significant (R^2=0.38*)* correlation between root length and a drought damage index among all genotypes under control (Fig 5.1A). However, a highly significant and negative (R^2=0.78; R^2= 0.67, *P<0.05*; Fig 5.1C) relationship was seen between root length of plants exposed to deficit (25% and 12% field capacity) irrigation and a drought damage index (Fig 5.2B & C).

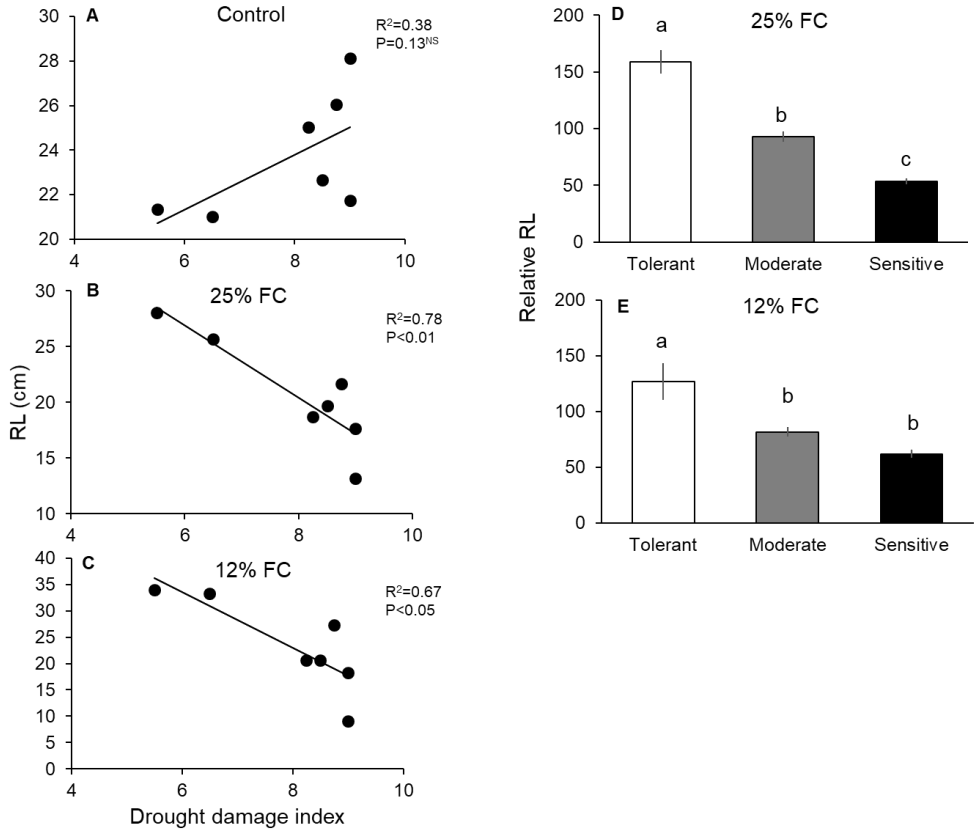

Figure 5.1 Correlations between drought damage index and root length (RL) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) RL values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at $P<0.05$

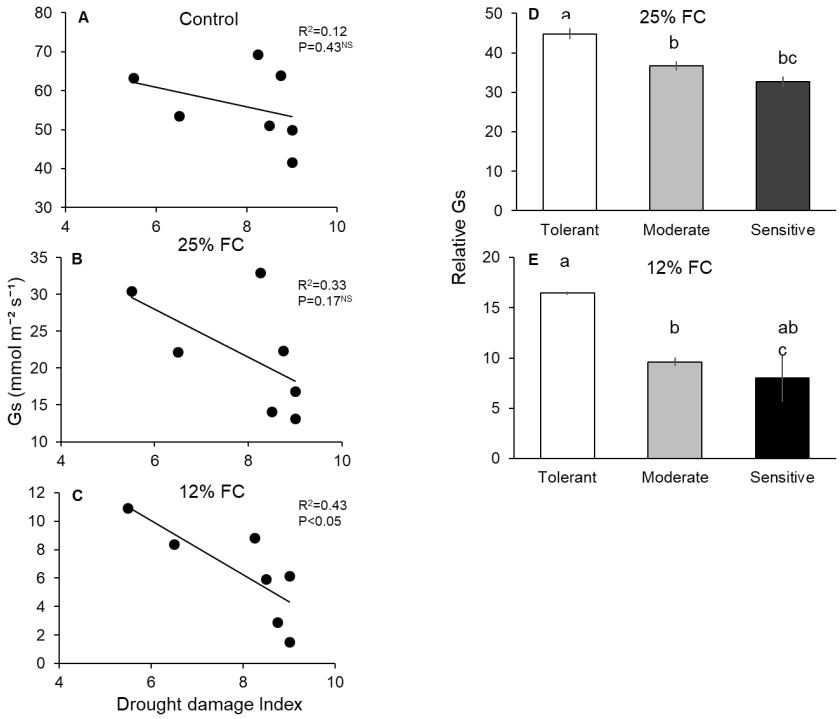

Figure 5.2 Correlations between drought damage index and stomatal conductance (Gs) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) Gs values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.2 Stomatal conductance (Gs)

Drought stress significantly reduced stomatal conductance of all genotypes relative to control plants. Under 25% field capacity irrigation, the reduction in Gs varied between 56% and 68% in all genotypes (Fig 5.2D). The reduction in Gs was more severe for plants grown under 12% field capacity irrigation, ranged between 84% and 93% as compared to plants grown under control conditions (Fig 5.2E). The stomatal conductance of the drought tolerant group remained higher than that of the moderate and sensitive group under these conditions (Fig 5.2E). A negative but non-significant correlation was found between stomatal conductance and drought damage index of plants under control and 25% field capacity irrigation conditions (R^2=0.12 and R^2=0.33; Fig 5.2A &B). However, a negative and significant (R^2=0.43, significant at *P<0.05)* correlation between stomatal conductance and drought damage index was found for plants grown under 12% field capacity irrigation (Fig 5.2C).

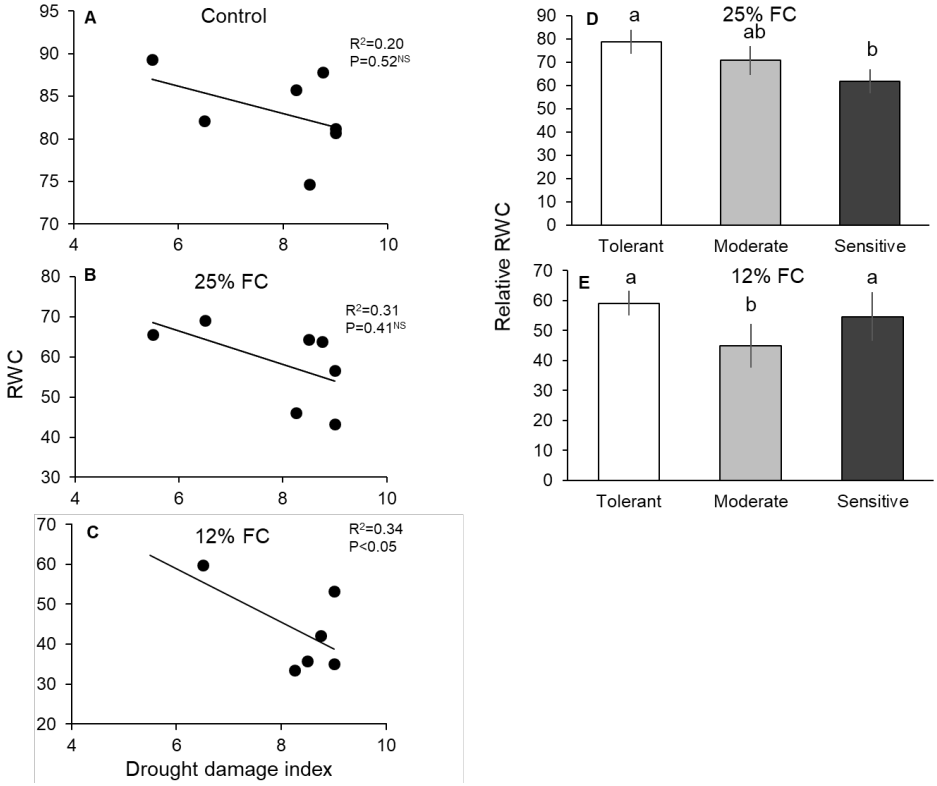

Figure 5.3 Correlations between drought damage index and relative water content (RWC) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) RWC values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.3 Relative water content (RWC)

Relative water content of all genotypes was higher under well-watered conditions and significantly reduced under water stress conditions. Under 25% field capacity irrigation, the relative water content of tolerant genotypes was reduced by 22% relative to control, whereas the genotypes in sensitive group exhibited 39% reduction (Fig 5.3D). Under severe drought (12% field capacity irrigation regime), tolerant group showed 59% reduction in the relative water content followed by sensitive genotypes (54%). The moderate tolerant group exhibited 44% reduction in the relative water content (Fig 5.3E). No significant correlation was observed between drought damage index and relative water content for plants grown under control and 25% field capacity (Fig 5.3A & B). However, a negative and significant (R^2=0.34*,* significant at *P<0.05*)

correlation was found for plants exposed to 12% field capacity irrigation and drought damage index (Fig 5.3C).

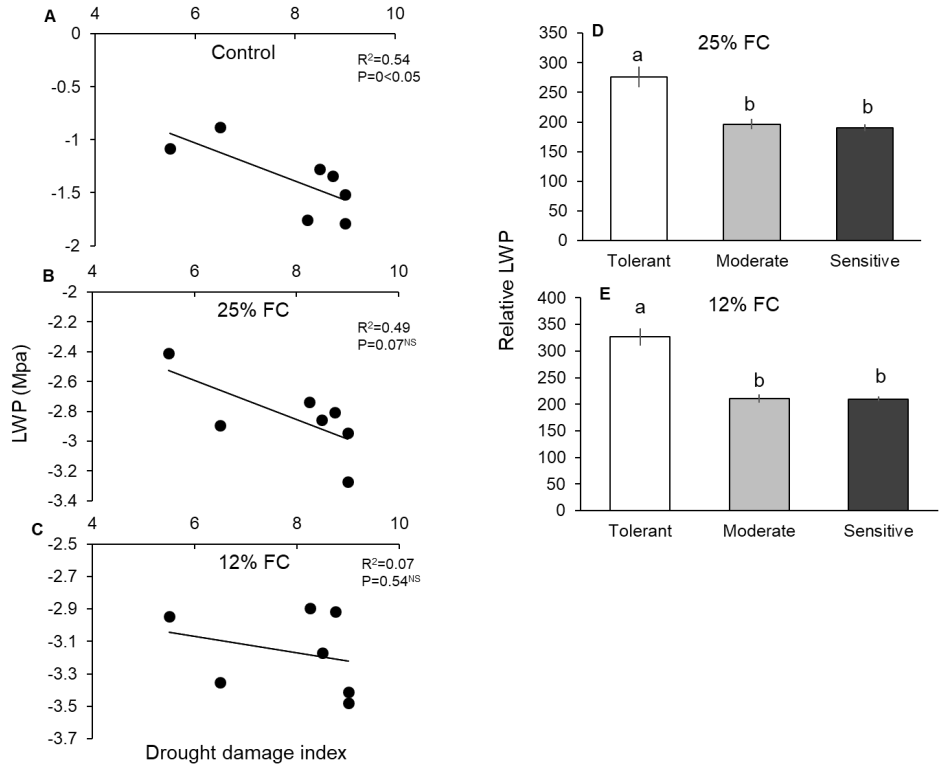

Figure 5.4 Correlations between drought damage index and leaf water potential (LWP) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) LWP values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.4 Leaf water potential (LWP)

The LWP of the drought stressed plants was significantly lower as compared to plants grown under control conditions reaching minimal values ranging between -2.41 and -3.27 MPa under 25% field capacity. The relative values of LWP increased between 90% and 175% for plants exposed to 25% field capacity. The tolerant group exhibited the maximum and sensitive group the minimum values (Fig 5.4D). Plants grown under severe drought stress (12% field capacity condition) exhibited more negative LWP values relative to plants grown under irrigated conditions (Fig 5.4E). The relative increase in leaf water potential ranged between 109% and 226%. A strong negative

correlation was found between leaf water potential and drought damage index of plants grown under control conditions (R^2=0.54; significant at *P<0.05*) (Fig 5.4A). A non-significant negative relationship was found between drought damage index and leaf water potential of plants grown under 25% field capacity irrigation (Fig 5.4B). However, no correlation was seen for LWP of plants grown under 12% field capacity conditions and drought damage index (Fig 5.4C).

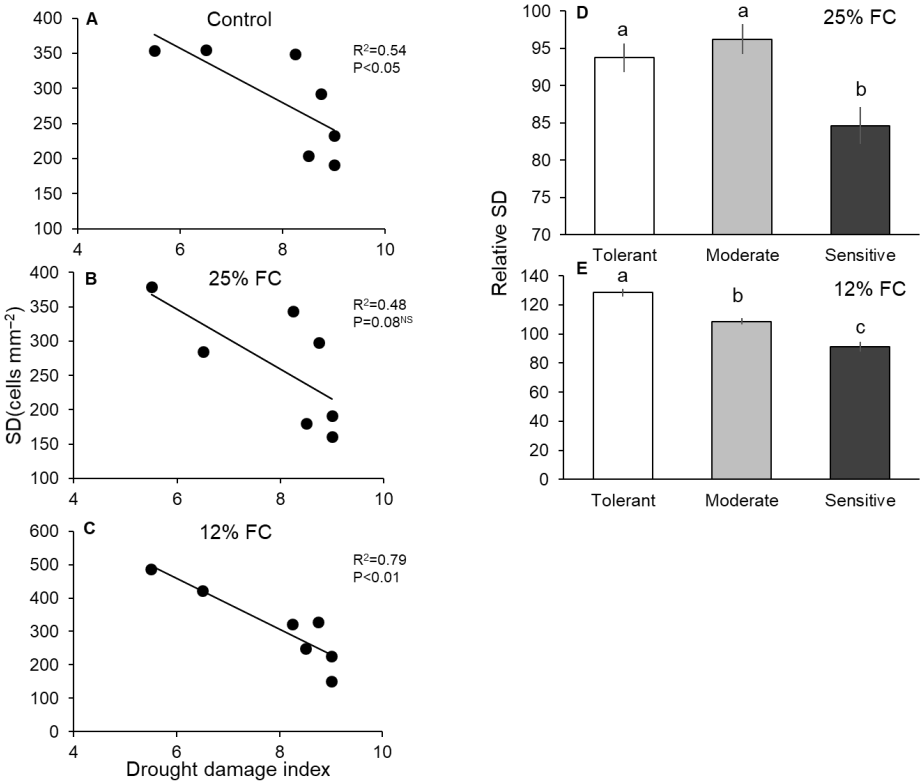

Figure 5.5 Correlations between drought damage index and stomatal density (SD) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) SD values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.5 Stomatal density (SD)

Stomatal density for all genotypes varied significantly under mild and severe water deficit. Under 25% field capacity conditions, all genotypes in three groups showed a decrease in SD ranged between 4% and 16% relative to control (Fig 5.5D). Under 12% field capacity irrigation, a noticeable increase (28%) in SD was found in tolerant group followed by 8% in a moderate group (Fig 5.5E). A strong negative correlation was found between damage index and stomatal density of plants grown under irrigated conditions (R^2=0.54; significant at *P<0.05*) (Fig 5.5A). However, a non-significant negative (R^2=0.48) relationship was observed between drought damage index and stomatal density of plants grown under 25% field capacity irrigation regime (Fig 5.5B). A very strong and negative correlation was seen between drought damage index and SD of plants exposed to 12% field capacity (R^2=0.79; significant at *P<0.01*) (Fig 5.5C).

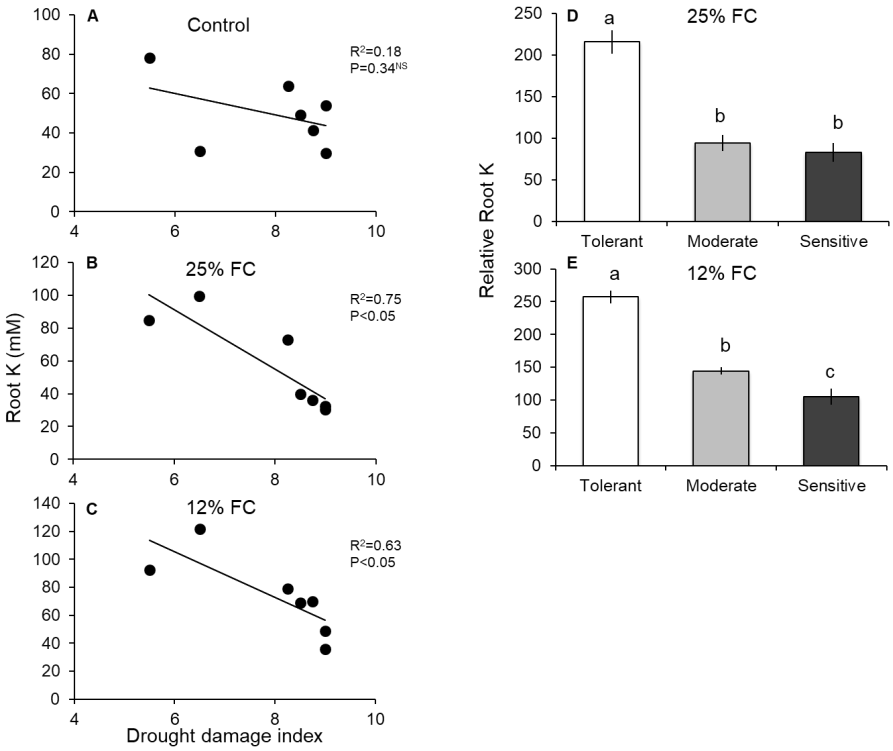

Figure 5.6 Correlations between drought damage index and root potassium (K) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) root K values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.6 Root K^+

Under 25% field capacity irrigation regime, all genotypes exhibited significant differences for root K relative to control (Fig 5.6D). In tolerant genotypes, root K concentration increased to 115% relative to control. However, sensitive and moderate tolerant group showed a significant decrease by 6% and 18% in root K. Under 12% field capacity conditions, all groups showed remarkable increase in root K concentration ranged between 5% and 157% relative to control (Fig 5.6E). There was a negative ($R^2=0.18$) but non-significant correlation found between drought damage index and root K of plants grown under control conditions (Fig 5.6A). A strong negative correlation was found between drought damage index and root K of plants exposed to 25% and 12% field capacity conditions (R^2=0.75 and R^2=0.63, significant at $P<0.05$) (Fig 5.6B & C).

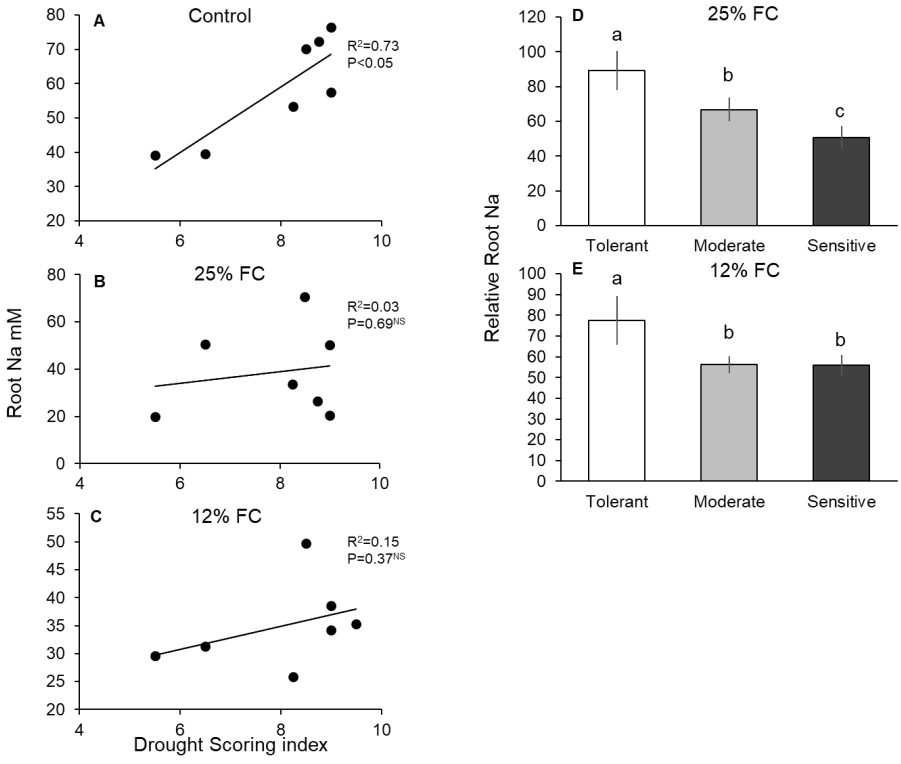

Figure 5.7 Correlations between drought damage index and root sodium (Na) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) root Na values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at $P<0.05$

5.2.7 Root Na^+

Drought induced a dramatic decrease in root Na^+ content of plants grown under mild and severe water deficit. Under 25% field capacity irrigation, all genotypes showed a decrease in Na concentration ranged by 11% to 50% compared to control (Fig 5.7D). However, plants exposed to severe drought stress (12% field capacity conditions) exhibited more noticeable decrease in root Na, ranging between 23% (tolerant varieties) and 45% (sensitive varieties) (Fig 5.7E). A positive correlation was found between damage index and root Na of plants grown under control conditions (R^2=0.73; significant at *P<0.05*) (Fig 5.7A). However, no significant correlation was found between drought damage index and root Na concentration of plants exposed to 25% and 12% field capacity irrigation (Fig 5.7B & C).

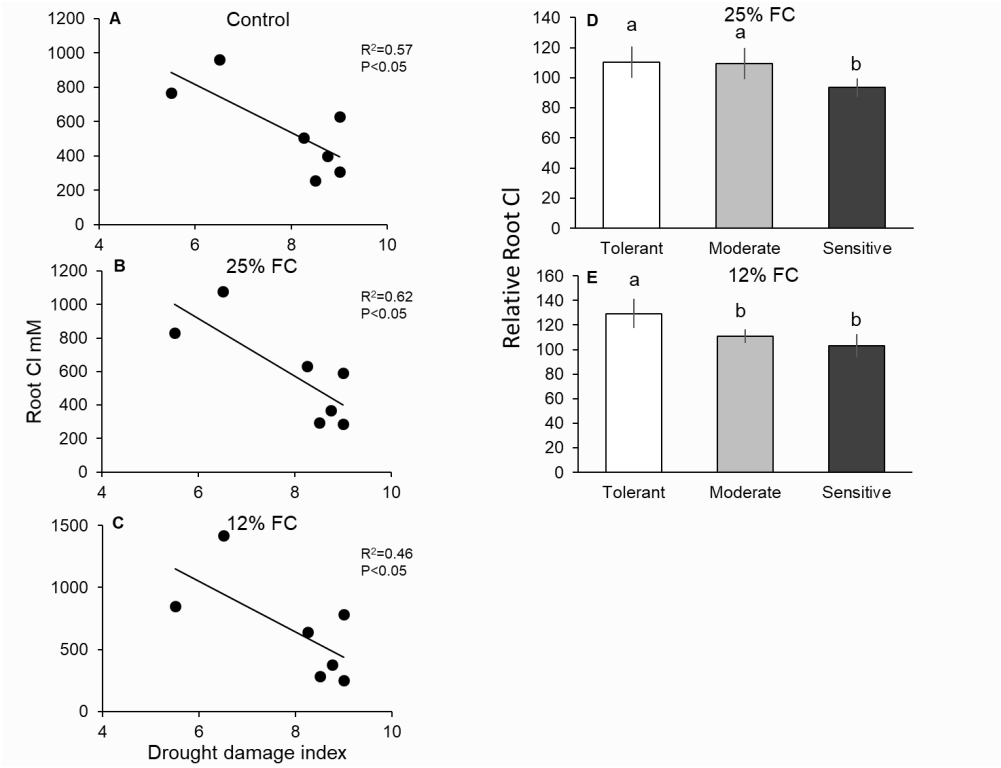

Figure 5.8 Correlations between drought damage index and root chloride (Cl) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) root Cl values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.8 Root Cl^-

Under 25% field capacity irrigation, tolerant genotypes exhibited root Cl content increased by 10% in tolerant group but decreased by 7% in sensitive group (Fig 5.8D). Under 12% field capacity irrigation, all genotypes showed an increase in root Cl concentration ranged between 3% and 29% as compared to control (Fig 5.8E). A strong negative correlation was found between drought damage index and root Cl of plants grown under control, 25% and 12% field capacity irrigation conditions (R^2=0.57; R^2=0.62; R^2=0.46: significant at *P<0.05*) (Fig 5.8A, B & C).

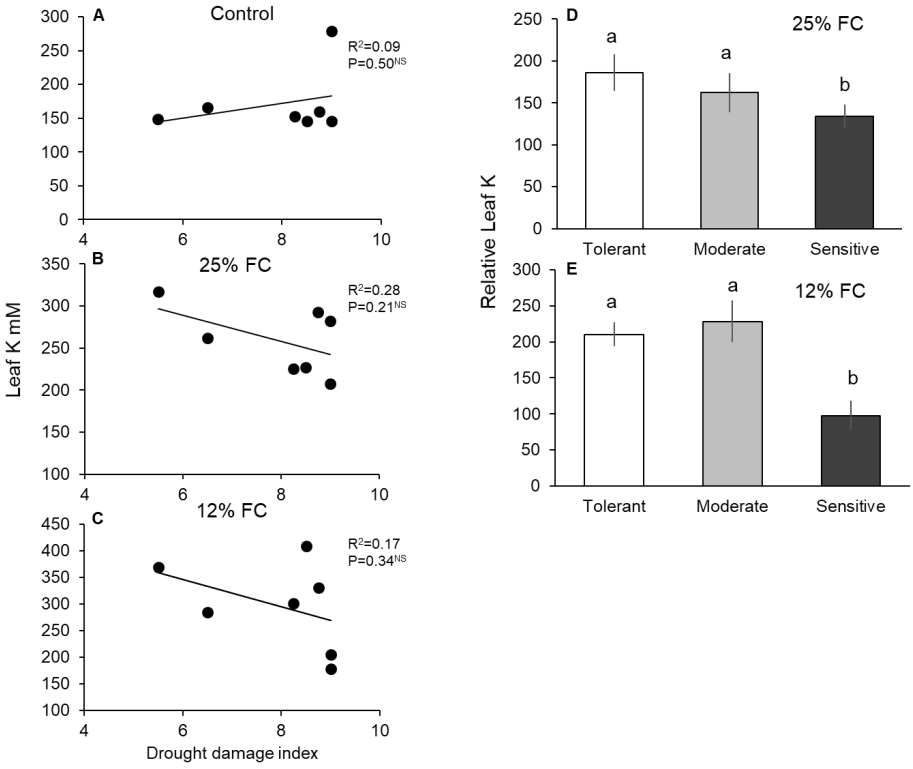

Figure 5.9 Correlations between drought damage index and leaf potassium (K) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) leaf K values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.9 Leaf K^+

Under 25% field capacity irrigation, the relative leaf K content varied between 86% and 33% of that in control, in the following order: tolerant>moderate>sensitive (Fig 5.9D). However, a substantial increase in leaf K (between 110% and 128% of control) was observed in moderate and tolerant genotypes grown under 12% field capacity irrigation conditions (Fig 5.9E). No correlation was found between drought damage index and leaf K of plants grown under control conditions (Fig 5.9A). A negative but non-significant correlation was seen between drought damage index and leaf K of plants grown under 25% and 12% field capacity irrigation (R^2=0.28, R^2=0.17) (Fig 5.9B & C).

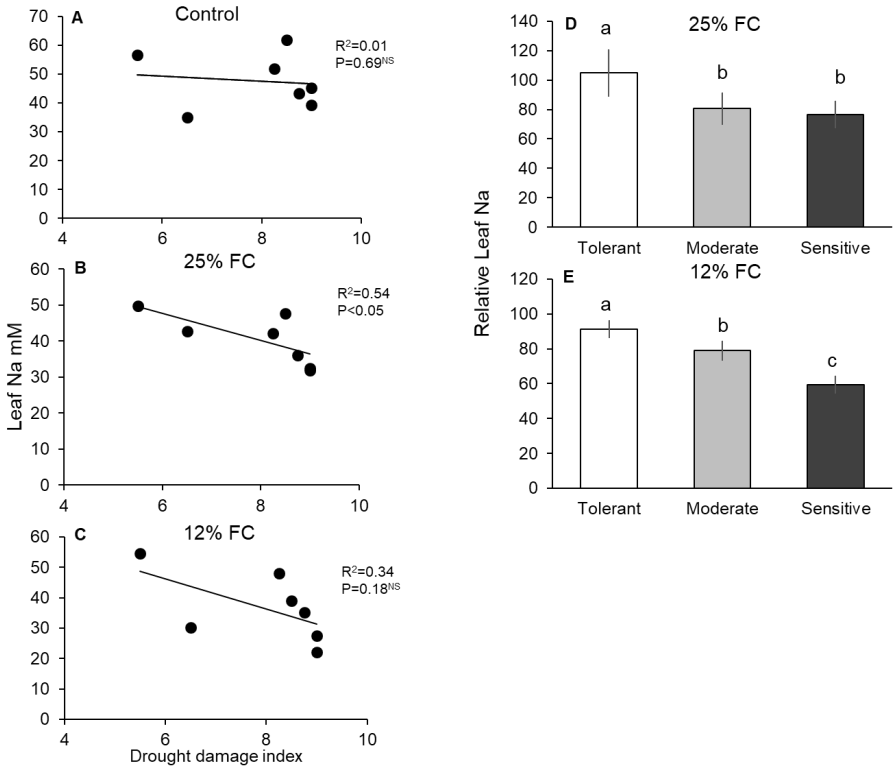

Figure 5.10 Correlations between drought damage index and leaf sodium (Na) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) leaf Na values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.10 Leaf Na^+

Both drought deficit irrigations exerted a dramatic decrease in leaf Na of all the genotypes except leaf Na in tolerant group under 25% field capacity conditions. Under 25% field capacity irrigation, Na content in tolerant genotypes slightly increased up to 4% while sensitive and moderate tolerant genotypes showed a decrease by 24% and 20% in leaf Na relative to control (Fig 5.10D). Under 12% field capacity irrigation, the highest reduction in leaf Na was exhibited by sensitive genotypes (41%) and the least by tolerant group (9%) (Fig 5.10E). No correlation was found between drought damage index and leaf Na of plants grown under control conditions (Fig 5.10A). However, a strong negative correlation was found between drought damage index and leaf Na of plants grown under 25% field capacity irrigation regime (R^2=0.54; significant at $P<0.05$) (Fig 5.10B). A negative (R^2=0.34) but non-significant correlation was observed between drought damage index and leaf Na of plants grown under severe drought stress (Fig 5.10C).

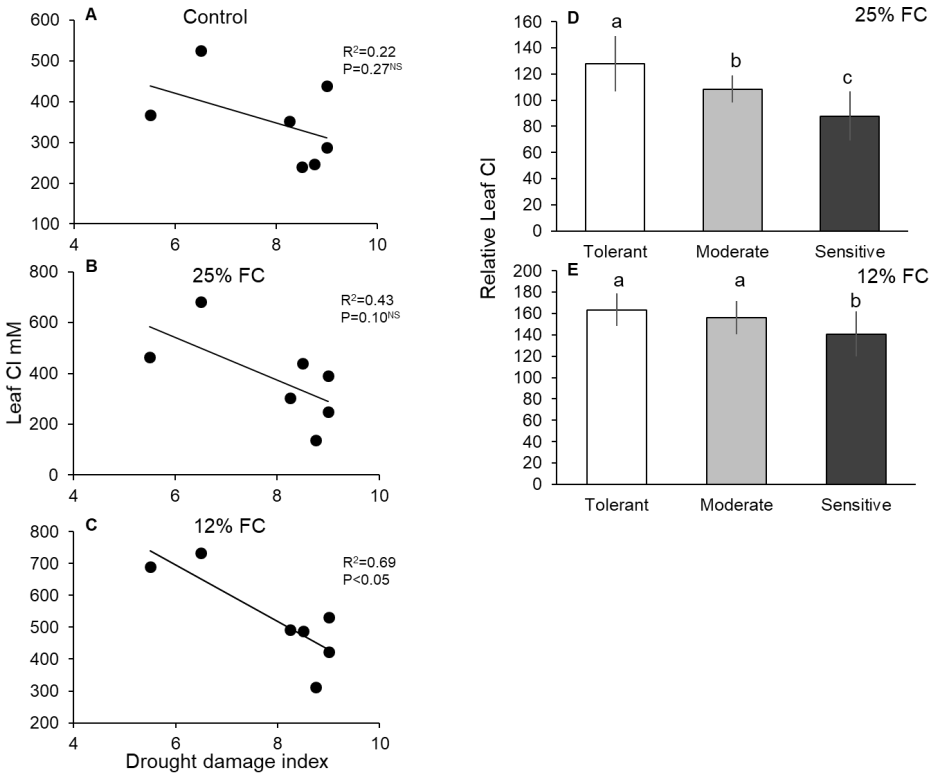

Figure 5.11 Correlations between drought damage index and leaf chloride (Cl) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) leaf Cl values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.11 Leaf Cl^-

Under 25% field capacity conditions, moderate tolerant and tolerant genotypes exhibited an increase by 8% to 27% in leaf chloride content compared to control. However, sensitive genotypes exhibited a decrease in chloride by 13% (Fig 5.11D). Under 12% field capacity irrigation regime, all the three groups showed a noticeable increase in leaf Cl content, by 40% to 63% (Fig 5.11E). A negative (R^2=0.22; R^2=0.43) but non-significant correlation was found between drought damage index and leaf Cl of plants exposed to control and 25% field capacity conditions (Fig 5.11A & B). However, a strong negative and significant correlation was found between drought damage index and leaf Cl of plants grown under 12% field capacity conditions (R^2=*0.*69; significant at *P<0.05*) (Fig 5.11C).

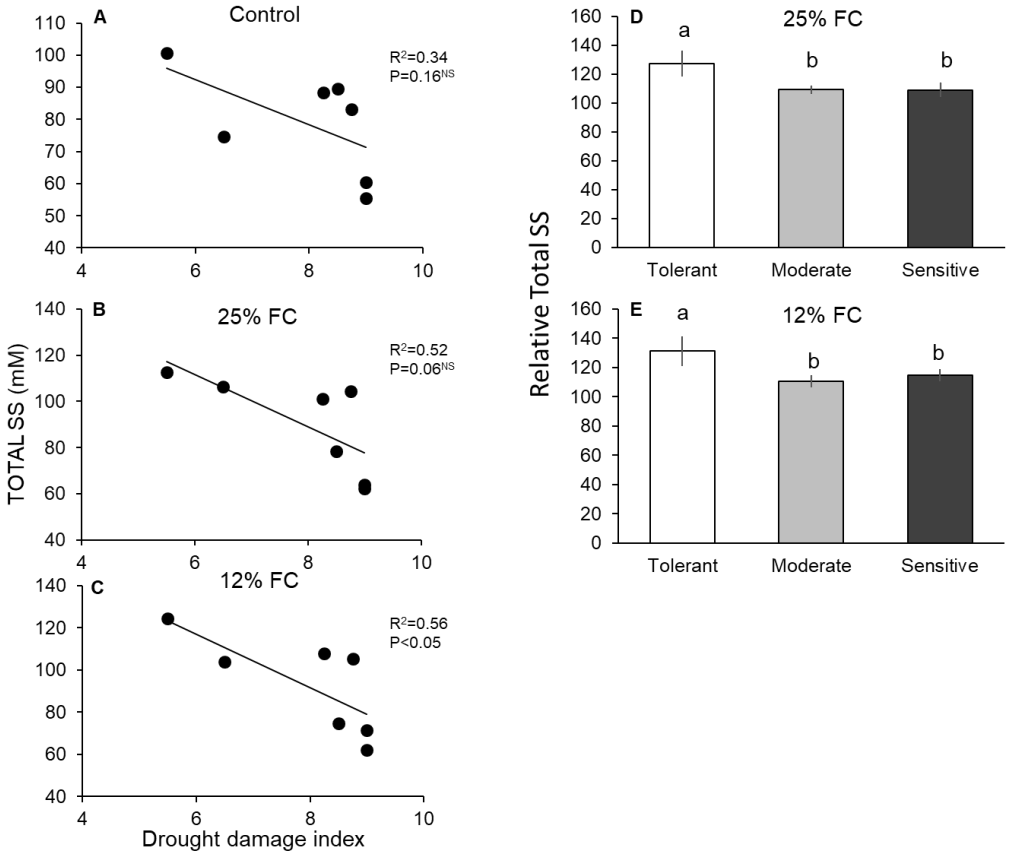

Figure 5.12 Correlations between drought damage index and total soluble sugars (SS) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) total SS values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.12 Total soluble sugars

Under mild drought stress (25% field capacity conditions), all the genotypes exhibited an increase in accumulation of total soluble sugars by 9% to 27% (Fig 5.12D). However, under severe drought stress (12% field capacity conditions), the tolerant genotypes exhibited 10% increase in total soluble sugars and sensitive was ranged between 10% and 31% (Fig 5.12E). Tolerant group showed the highest increase whereas the sensitive group exhibited the least. A non-significant negative (R^2=0.34; R^2=0.52 respectively) correlation was found between drought damage index and total soluble sugars of plants grown under control and 25% field capacity conditions (Fig 5.12A & B). However, a strong negative and significant relationship was found

between drought damage index and total soluble sugars of plants grown under 12% field capacity conditions (R^2=0.56; significant at *P<0.05*) (Fig 5.12C).

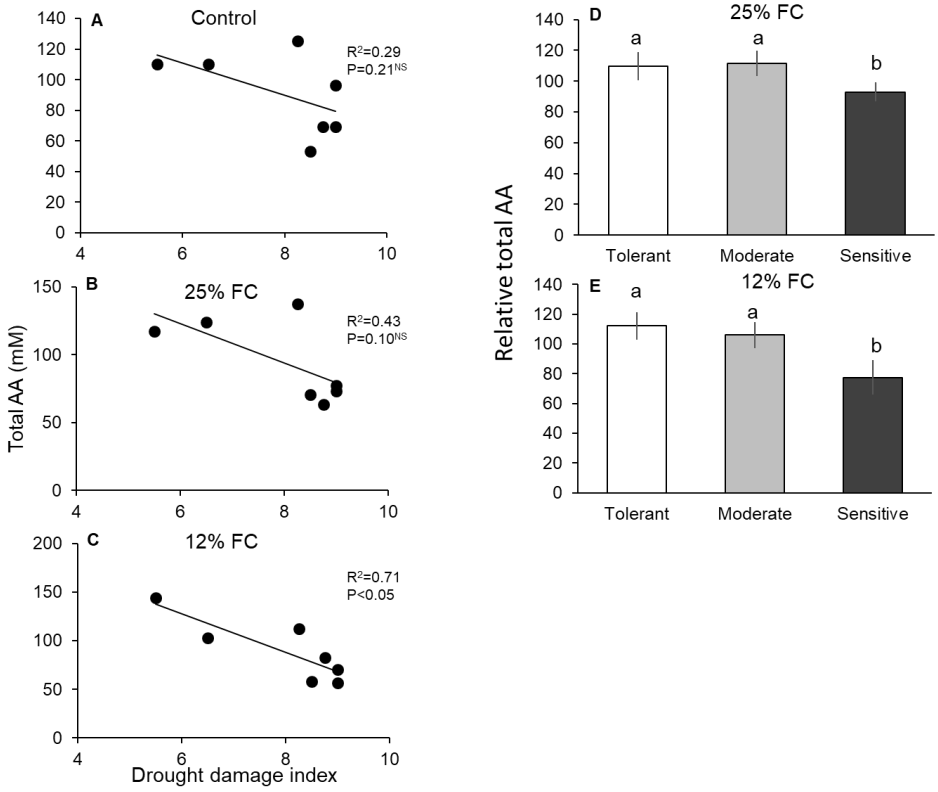

Figure 5.13 Correlations between drought damage index and total amino acids (AA) of plants grown under control (A), 25% field capacity, (B) and 12% field capacity (C) deficit irrigation conditions. Each point represents a separate variety. Panels D and E show relative (% of control) total AA values of three clusters of barley genotypes (tolerant, moderate, and sensitive to drought) for plants grown under deficit irrigation conditions (25% and 12% full field capacity, respectively). Different lowercase letters indicate the significance difference between clusters at *P<0.05*

5.2.13 Total amino acids

Drought stress altered total amino acids concentration in all barley genotypes relative to control. Under 25% field capacity conditions, tolerant and moderate tolerant groups showed an increase of 10% in total amino acids relative to control. However, sensitive genotypes exhibited a reduction of 8% in amino acid concentrations relative to control (Fig 5.13D). Under 12% field capacity irrigation regime, tolerant and moderate groups exhibited an increase by 5% and 12%, respectively, in total amino acids relative to control; in sensitive genotypes the total amino acids decrease by 13% (Fig 5.13E). The correlation analysis showed a non-significant negative correlation (R^2=0.29; R^2=0.43) for drought damage index and total amino acids of plants grown under control and 25% field capacity conditions (Fig 5.13A & B). However, a strong negative and highly significant correlation was seen between damage index and total amino acids of plants exposed to 12% field capacity conditions (R^2=0.71; significant at $P<0.05$) (Fig 5.13C).

5.2.14 Leaf osmolality

Under control conditions, the average leaf osmolality for barley genotypes ranged between 1229 mmol/kg and 903 mmol/kg (Table 5.1). Variety Numar had the highest and Fleet had the lowest osmolality. The highest percent contribution of inorganic osmolytes was exhibited by Franklin (62%) and Commander showed the highest contribution of organic osmolytes (22%). Under 25% field capacity irrigation, leaf osmolality was ranged between 1532 mmol/kg and 892 mmol/kg (Table 5.2). The highest osmolality was found in Numar and the lowest in Franklin. The highest percent contribution of inorganic osmolytes in leaf osmolality was exhibited by Numar (64%). However, Commander showed the highest percent contribution of inorganic osmolytes (19%). The largest contribution to tissue osmolality was by the accumulation of Cl followed by K and TAA. Na was the least contributor to leaf osmolality under 25% field capacity irrigation regime. Under 12% field capacity conditions, leaf osmolality varied between 1783 mmol/kg and 1167 mmol/kg (Table 5.3). Numar had the highest osmolality and Gairdner the lowest. The relative contribution of inorganic and organic osmoles towards the leaf osmolality was in the order Cl > K > TSS > TAA > Na.

Table 5.1 Contribution of inorganic ions (K+, Na+, Cl-) and organic osmolytes towards the osmotic adjustment in barley leaves grown under control conditions. TSS = total soluble sugar; TAA = total amino acids

Variety	Osmolality measured	Concentration measured					Relative contribution to osmotic adjustment					
	mmol kg^{-1}	K^+ mM	Na^+ mM	Cl^- mM	TSS mM	TAA mM	K^+	Na^+	Cl^-	TSS	TAA	Others
Tolerant												
Numar	1229	165	35	525	75	110	13	3	43	6	9	26
ZUG293	1023	148	57	368	101	110	15	6	36	10	11	23
Moderately tolerant												
Commander	934	153	52	352	88	125	16	6	38	9	13	18
Fleet	903	146	62	240	90	53	16	7	27	10	6	35
X123	1021	160	43	246	83	69	16	4	24	8	7	41
Sensitive												
Franklin	992	279	45	287	60	69	28	5	29	6	7	25
Gairdner	1123	146	39	439	56	97	13	4	39	5	9	31

Table 5.2 Contribution of inorganic ions (K^+, Na^+, Cl^-) and organic osmolytes towards the osmotic adjustment in barley leaves grown under deficient irrigation (25% of full field capacity). TSS = total soluble sugar; TAA = total amino acids

Variety	Osmolality measured	Concentration measured					Relative contribution to osmotic adjustment					
	mmol kg^{-1}	K^+ mM	Na^+ mM	Cl^- mM	TSS mM	TAA mM	K^+	Na^+	Cl^-	TSS	TAA	Others
Tolerant												
Numar	1532	262	43	681	106	124	17	3	44	7	8	21
ZUG293	1367	317	50	464	113	118	23	4	34	8	9	22
Moderately tolerant												
Commander	1277	225	42	303	101	137	18	3	24	8	11	37
Fleet	1458	227	48	439	78	71	16	3	30	5	5	41
X123	966	293	36	138	104	63	30	4	14	11	7	34
Sensitive												
Franklin	892	208	33	250	62	73	23	4	28	7	8	30
Gairdner	1138	282	32	390	64	77	25	3	34	6	7	26

Table 5.3 Contribution of inorganic ions (K^+, Na^+, Cl^-) and organic osmolytes towards the osmotic adjustment in barley leaves grown under deficient irrigation (12% of full field capacity). TSS = total soluble sugar; TAA = total amino acids

Variety	Osmolality measured	Concentration measured					Relative contribution to osmotic adjustment					
		K^+	Na^+	Cl^-	TSS	TAA	K^+	Na^+	Cl^-	TSS	TAA	Others
	mmol kg^{-1}	mM	mM	mM	mM	mM						
Tolerant												
Numar	1783	285	30	733	104	103	16	2	41	6	6	30
ZUG293	1662	369	55	689	124	144	22	3	41	7	9	17
Moderately tolerant												
Commander	1455	302	48	492	108	112	21	3	34	7	8	27
Fleet	1592	409	39	487	75	58	26	2	31	5	4	33
X123	1255	330	35	311	105	83	26	3	25	8	7	31
Sensitive												
Franklin	1216	205	22	532	71	56	17	2	44	6	5	27
Gairdner	1167	178	28	423	62	71	15	2	36	5	6	35

5.2.15 Root osmolality

Under control conditions, root osmolality varied between 1582mmol/kg and 762mmol/kg (Table 5.4). The highest root osmolality was found in Numar (1582mmol/kg) followed by ZUG293 (1045mmol/kg) while the lowest osmolality was found in Fleet (762mmol/kg). The contribution of inorganic osmolytes ranged between 86% (ZUG293) and 51% (Gairdner). Under 25% field capacity, the root osmolality ranged between 1634 mmol/kg and 623 mmol/kg (Table 5.5). The average pattern of % contribution by K, Na, and Cl was the same as observed in leaf (Cl>K>Na). The root osmolality of plants exposed to severe stress (12% field capacity irrigation regime) varied between 2043 mmol/kg and 688 mmol/kg (Table 5.6). The percent contribution by K, Na, and Cl to root osmolality varied between 78% to 39%. Inorganic osmolytes made the highest contribution to osmotic adjustment in cultivar Numar, while their contribution was lowest in cultivar Gairdner.

Table 5.4 Contribution of inorganic ions (K+, Na+, Cl-) towards the osmotic adjustment in barley roots grown under control conditions

Variety	Osmolality measured	Concentration measured			Relative contribution to osmotic adjustment			
	mmol kg^{-1}	K^+ mM	Na^+ mM	Cl^- mM	K^+	Na^+	Cl^-	Others
Tolerant								
Numar	1582	121	40	961	8	3	61	29
ZUG293	1045	90	39	766	9	4	73	14
Moderately tolerant								
Commander	895	64	53	506	7	6	57	30
Fleet	762	49	70	259	6	9	34	50
X123	934	41	72	367	4	8	39	49
Sensitive								
Franklin	932	30	58	629	3	6	67	23
Gairdner	870	54	76	310	6	9	36	49

Table 5.5 Contribution of inorganic ions (K^+, Na^+, Cl^-) towards the osmotic adjustment in barley roots grown under deficient irrigation (25% of full field capacity)

Variety	Osmolality measured	Concentration measured			Relative contribution to osmotic adjustment			
	mmol kg^{-1}	K^+ mM	Na^+ mM	Cl^- mM	K^+	Na^+	Cl^-	Others
Tolerant								
Numar	1634	99	50	1078	6	3	66	25
ZUG293	1227	85	20	832	7	2	68	24
Moderately tolerant								
Commander	984	73	34	633	7	3	64	25
Fleet	802	40	71	296	5	9	37	49
X123	834	36	26	284	4	3	34	58
Sensitive								
Franklin	982	32	20	593	3	2	60	34
Gairdner	623	30	50	287	5	8	46	41

Table 5.6 Contribution of inorganic ions (K^+, Na^+, Cl^-) towards the osmotic adjustment in barley roots grown under deficient irrigation (12% of full field capacity)

Variety	Osmolality Measured	Contribution Osmoles			Relative contribution to osmotic adjustment			
	mmol kg^{-1}	K^+ mM	Na^+ mM	Cl^- mM	K^+	Na^+	Cl^-	Others
Tolerant								
Numar	2043	122	31	1421	6	2	70	23
ZUG293	1672	92	30	849	6	2	51	42
Moderately tolerant								
Commander	1125	79	26	643	7	2	57	34
Fleet	688	69	50	286	10	7	42	41
X123	1078	70	35	376	6	3	35	55
Sensitive								
Franklin	1322	36	39	783	3	3	59	35
Gairdner	872	49	34	253	6	4	29	61

5.2.16 Principle component analysis

To better understand the relationships, similarities and dissimilarities among the indicators of drought tolerance, principle component analysis was conducted based on whole plant trait data of seven barley genotypes grown under 12% field capacity conditions (Fig 5.14). This analysis revealed that the two first PCA explained cumulative variance of 76.09%. The principle component 1 (PC1) explained 55.73% of the variation and exhibited a negative correlation with leaf Cl, root Cl, Gs, TAA, SD, root K, RL, TSS, leaf Na and a positive correlation with root Na. Hence, the first dimension can be referred as the best indicator of drought tolerance. The genotypes with higher values of PC1 are expected to be drought tolerant. The principle component 2 (PC2) describes 20.36% of total variability with negative correlative with leaf K, positive correlation with RWC and no significant correlation with LWP. The genotypes with higher values of PC2 and smaller values of PC1 were identified as moderate tolerant; the genotypes with lower values of PC1 and PC2 are described as drought susceptible genotypes.

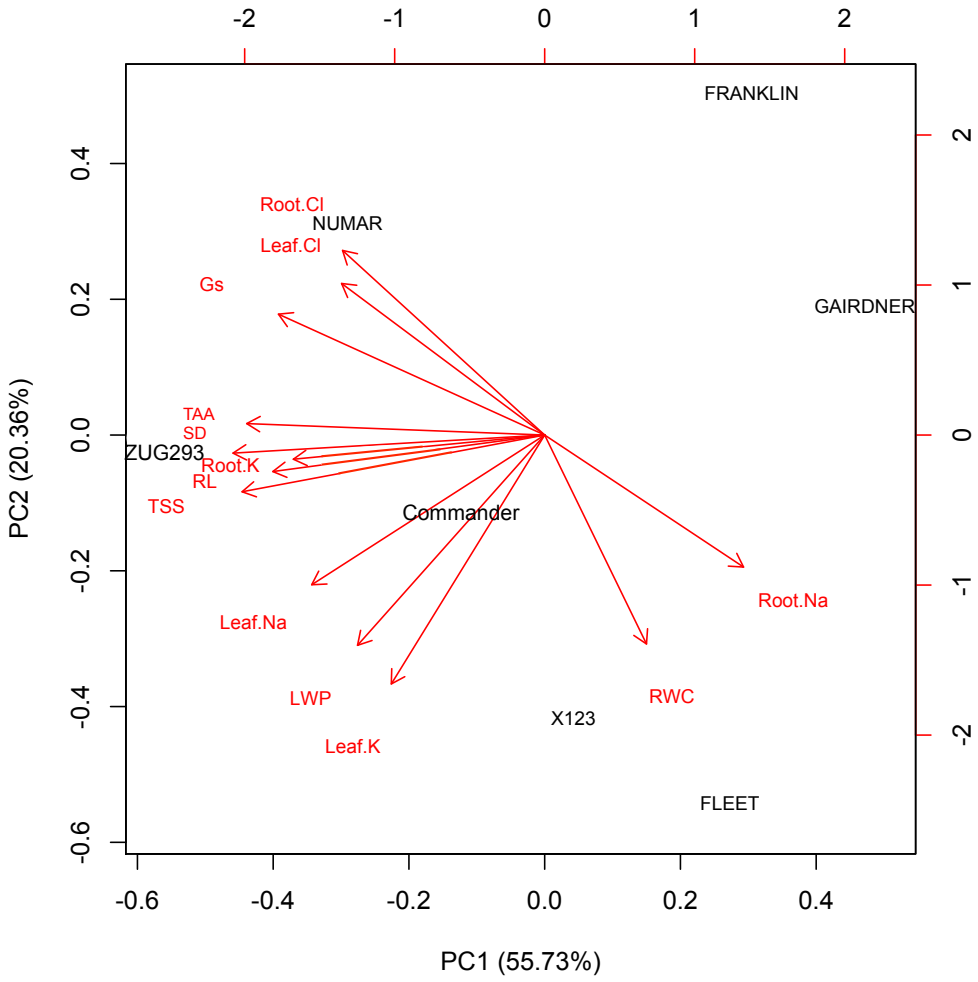

Figure 5.14 Biplot for drought tolerance indices in seven barley genotypes based on first two components measured in plants grown under 12% field capacity. RL: Root length; Gs: Stomatal conductance; SD: Stomatal density; RWC: Relative water content; LWP: Leaf water potential: Root K; Root Na; Root Cl; Leaf K; Leaf Na; Leaf Cl; TSS: Total soluble sugars and TAA: Total amino acids.

5.3 Discussion

5.3.1 Stomatal conductance is clustered together with root and leaf Cl

PCA analysis revealed (Fig 5.14) that Gs is clustered with root and leaf chloride, suggesting a role of Cl^- in the regulation of stomatal aperture. Chloride can be used as the counterion for K during stomatal opening and has been found to reduce malate production in guard cells (Van Kirk and Raschke, 1978). Thus, under drought conditions, where energy is a limiting factor for plants, it is likely that Cl acts as a beneficial nutrient that limits the energy costs associated with malate biosynthesis and stomatal opening. Consistent with this view, previous studies have found that treating plants with optimum Cl increases stomatal conductance (Chen et al., 2013). The data also suggest that the observed increase in leaf Cl is associated with increased Cl uptake in roots. Under optimal conditions, root K uptake is passive while chloride uptake is an active process occurring via a $2H^+$ /Cl symporter (Sparks, 2012). However, when exposed to drought, root plasma membrane depolarisation will limit passive K uptake (Shabala et al., 2005) but at the same time decrease the steepness of electric gradient preventing Cl^- entry (Broadley, 2012). Hence, plants could rely more heavily on Cl than K for cell osmoregulation (including in guard cells).

5.3.2 Root length correlate with root K, total amino acids and total soluble sugars of leaves

Root length is strongly associated with root K, total soluble sugars, and amino acids (Fig 5.14). Elongation of root length under drought conditions enable plants to extract water and nutrients from deeper soil layers (Hu and Schmidhalter, 2005). The increase in root length consequently improved the rate of K^+ diffusion from the soil matrix towards the roots (Wang et al., 2013). Moreover, the adequate supply of K is also essential in translocation of photoassimilates to sustain root growth (Romheld and Kirkby, 2010). The accumulation of K, total soluble sugars and amino acids is associated with active osmotic adjustment under drought stress conditions. Turgor maintenance due to osmotic adjustment enhance root growth during water deficit (Khanna, 1990). Elongation of lateral roots requires sugars as an energy resource and

as a substrate for synthesis of components of the cell and the cell wall (Ogawa et al., 2005). Soluble sugars substantially contribute to the initiation and elongation of lateral roots and thereby maintain lateral root growth (Ogawa, 1996; Ogawa et al., 2005). Root growth increases as a result of significant accumulation of free amino acids (Yang et al., 2015) that can be used as a nitrogen sources, in addition to their possible role in osmotic adjustment.

5.3.3 Sensitive varieties have more negative LWP under control conditions

Sensitive varieties had more negative leaf water potential as compared to tolerant genotypes in well-watered conditions (Fig 5.4). LWP and relative water content are closely related to each other. The reason for low water potential in sensitive varieties could be due to their low relative water content in control conditions (Fig 5.3). A reduction of only 5% relative water content could resulted in a decrease of approximately 14.4%-36.9% of water potential (Gholami et al., 2012). Therefore, genotypes with more decline in RWC are not capable of maintain internal water status and maintaining hydration of protoplasm could cause more negative leaf water potential. The increase in transpiration rate and low water use efficiency could also be the factors effecting more negative leaf water potential (Snyman et al., 1997).

5.3.4 Drought tolerance is associated with higher root K and Cl content

A significant correlation was found between root K and Cl content and drought tolerance, suggesting the critical role of inorganic nutrients in regulating different physiological processes of the plant under water deficit. Potassium plays a crucial role in improving water homeostatic by regulating cell turgor and osmotic adjustment under drought conditions (Hasanuzzaman et al., 2018). Cl as an osmotically active solute act as counterion with K to maintain membrane electroneutrality and its uptake also double the osmotic effect in the plants (Shabala and Shabala, 2011). Under water stress, the uptake of K and Cl from the roots increases and generally these inorganic ions are responsible for a very large and rapid turgor recovery (> 90%) (Shabala and Lew, 2002). The energy cost in uptake and compartmentation of inorganic ions is at least an order of magnitude lower than *de novo* synthesis of compatible solutes (Raven, 1985) and, thus, is preferred. The improved stomatal regulation by K and Cl facilitates

high rate of photosynthesis which consequently increase the plant biomass under drought conditions (Broadley, 2012; Egilla et al., 2001).

5.3.5 Drought tolerance is positively correlated to total sugars and amino acids

Water stress induced an accumulation of compatible solutes including total soluble sugars and amino acids in many crop plants (Babita et al., 2010; Mostajeran and Rahimi-Eichi, 2009; Templer et al., 2017). Drought tolerant genotypes have accumulated more sugars and amino acids to minimize the harmful effects of drought (Guo et al., 2018; Yamada et al., 2005). The accumulation of these compatible solutes increases the ability of cells to retain water without disturbing normal cellular functions. The beneficial role of sugars and amino acids also include stabilizing the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and detoxification of reactive oxygen species (Blum, 2017; Sengupta et al., 2016; Singh et al., 2015). Our results revealed that besides inorganic ions such as K and Cl, the accumulation of total soluble sugars and amino acids play a significant role in improving drought tolerance (Okçu et al., 2005; Pei et al., 2010).

5.3.6 Drought sensitive varieties accumulate more Na in the root

Under well-watered conditions, the highest accumulation of Na in roots was found in sensitive genotypes (Franklin and Gairdner) as compared to moderate and tolerant genotypes (Fig 5.7). Under stress conditions, the plasma membrane of the root epidermal cells is usually depolarized (Shabala et al., 2015), making thermodynamically-passive K^+ uptake impossible. As a result, plants need to rely on high affinity HAK/KUP uptake systems (Nieves-Cordones et al., 2014). This comes with the energy cost and is therefore less efficient. Stress-tolerant varieties usually maintain more negative membrane potential (Chen et al., 2007) and thus may not face this dilemma. Sensitive varieties which cannot allocate sufficient amount of ATP for H^+-ATPase pump operation, might opt to rely on using Na^+ instead of K^+, for osmotic adjustment purposes. Sodium is present in all soils at relatively high concentrations (mM range) and, thus, can be taken up passively, even under non-saline conditions.

5.3.7 Drought stress tolerance correlates with stomatal density

The leaf stoma act as a pivotal gate controlling the exchange of CO_2 between the interior of plant and atmosphere and diffusive water vapour during transpiration (Zhao et al., 2015). According to the results shown in Fig 5.5C, a positive correlation was found between stomatal density and drought stress tolerance. We noted a clear trend in stomatal density in three barley groups under drought stress (T>M>S). These results were unexpected, given the reported evidence that reducing SD results in improved water use efficiency (Caine et al., 2018; Hughes et al., 2017). However, the rate of water movement through the leaf will be determined not only by the number of stomata, but also their size and aperture. Under drought conditions stomata are generally small resulting in a decline in transpiratory water loss (Pearce et al., 2006; Sarker and Hara, 2011). Many studies showed that water deficit leads to decrease in stomatal size (Martínez et al., 2007; Quarrie and Jones, 1977; Xu and Zhou, 2008). A negative correlation between stomatal density and stomatal size was attained depending on reducing stomata length (Shipeng, 2006) or width (Zhang et al., 2006). The studies on ABA mediated signalling cascade in guard cells under drought showed that in response to ABA, plant species with bigger stomata closes them more slowly exhibiting lower drought sensitivity while in contrast small stomata can open and close more rapidly (Hartung et al., 2002; Schachtman and Goodger, 2008). The small stomata are generally associated with higher stomatal density regulation of stomatal conductance and consequently improving photosynthesis (Hetherington and Woodward, 2003; Royer, 2001).

5.3.8 No correlation was reported between drought tolerance and leaf K and Na content

Plant adaptation to drought requires mechanism dealing with osmotic adjustment in roots and/or leaves. The results of present study implied that osmotic adjustment by inorganic osmolytes in roots were attributed towards drought tolerance but there was no correlation of leaf K and Na with drought tolerance. The roots are the first organs exposed to soil water deficit which then send signals to shoots above ground (Janiak et al., 2015). Therefore, under water limited conditions, it is assumed that more rapid osmotic adjustment could occurs in the roots before leaves to enhance turgor pressure

for continued root growth and absorption of water and nutrients and consequently delay the onset of water stress in the shoot (Hsiao and Xu, 2000; Ogawa and Yamauchi, 2006). Drought tolerance was also positively correlated with total sugars (Fig 5.12C) and total amino acids (Fig 5.13C) which revealed that these genotypes might be relied more on organic compounds for tolerance as compared to inorganic ions. This is consistent with previous results (Anjum et al., 2017; Farooq et al., 2009b; Pawar et al., 2015) and is likely to be explained by important chaperon-like of ROS-scavenging function of some of these organic osmolytes (in addition to merely the osmotic adjustment). Hence, we could recommend breeders to select genotypes which are drought tolerant based on root related traits such as root K and Cl and shoot organic osmloyte content.

Chapter 6. Revealing mechanisms of osmoregulation and abscisic acid- mediated signaling in hyperosmotically-stressed barley roots

6.1 Introduction

Plants have evolved different mechanisms to deal with various environmental stresses, in order to maintain growth and development. The control of cell enlargement plays a crucial role in drought stress responses and plant growth (Basu et al., 2016; Maggio et al., 2006). Cell growth as a consequence of the cell expansion is modulated predominately by the turgor pressure; a physical force against the cell wall that is maintained by osmotic adjustment via osmotically active substances, such as inorganic ions uptake and sugars and amino acids (Osakabe et al., 2013). However, while the need for osmotic adjustment is universally accepted, the relative contribution of inorganic ions and (organic) compatible solutes have been debated in literature for many years and remains largely unresolved. It was a conventional belief that the prime role of compatible solutes is in osmotic adjustment. However, significant evidence has been accumulated suggesting that the function of compatible solutes is not limited by merely osmotic adjustment (Shabala and Shabala, 2011). The suggested roles include reactive oxygen species (ROS) scavenging, osmoprotection of key membrane transport proteins, and their role as a reservoir of carbon and nitrogen source (Bohnert and Jensen, 1996; Giri, 2011; Umezawa et al., 2006). If compatible solutes are not involved directly in cell osmoregulation, the only way for a plant cell to stabilize normal turgor pressure is via uptake of inorganic ions (mainly K^+, Na^+ and Cl^-). K plays a prime role in cell turgor maintenance, osmotic adjustment and aquaporin function under drought conditions (Wang et al., 2013; Waraich et al., 2012). In addition to K^+, Na^+ facilitate the growth and volume of plants if present in low concentrations (Blumwald, 2000). Besides K and Na, Cl acts as an osmotically active solute in the vacuole (Broadley et al., 2012; Flowers, 1988). To elucidate ionic mechanism of osmotic adjustment, various experiments have been performed in the past using non-invasive ion-selective microelectrode technique showing the fast turgor recovery and the increased uptake of K^+, Na^+ and Cl^- after the onset of hyperosmotic stress caused by mannitol treatment in elongation zone of Arabidopsis roots (Shabala

and Lew, 2002). Can this conclusion made on Arabidopsis roots be extrapolated to barley?

Plant roots can be divided longitudinally into anatomically distinct developmental zones namely apical meristem, elongation zone and mature zone, each of which have different abilities to take up and transport ions and water (Foster and Miklavcic, 2016). This differential responses of ions transport in different root zones has been previously indicated in response to different abiotic stresses particularly salinity (often called "physiological drought") in which application of salt treatment roots showed massive K^+ loss in elongation zone, which is 9-10 fold bigger compared to K^+ efflux from mature zone (Chen et al., 2005; Shabala et al., 2016). Nonetheless, no work has been done for mapping different root zones in response to hyperosmotic stress to explore that which root zone exhibited the major uptake of ions mainly K^+, Na^+ and Cl^-.

Osmotic stress causes rapid, significant, and prolonged hyperpolarization of plasma MP (Shabala and Lew, 2002; Teodoro et al., 1998; Zingarelli et al., 1999). It was believed that the major source for generating the MP in higher plant cells is the activity of the electrogenic ATP-dependent H^+ pump. The PM H^+-ATPases generate the proton motive force and thus are central to the maintenance of membrane potential (MP) and the channel mediated uptake of essential cations such as K^+ and Na^+ is increased (Sondergaard et al., 2004). Steeper H^+ gradients created by H^+ ATPase also lead to increased uptake of anion Cl^- as a result of increased driving force of proton coupled symport systems (Shabala and Lew, 2002). Although there is no available data, as root MP is the fundamental factor determining root ion transport and thus of paramount importance for cell turgor maintenance and growth, it is logical to expect that the osmotic induced changes in MP may be correlated with drought tolerance.

Plant hormones coordinate adaptive changes in cellular osmotic regulation. Abscisic acid (ABA) regulates various molecular events in response to water deficit stress and plant growth. The major functions of ABA accumulation in response to water stress involves modulation of stomatal aperture, which minimizes the loss of water through transpiration (Bauer et al., 2013; Lee and Luan, 2012). Therefore, it is thought to act as a signal for the initiation of regulatory processes involved in the adaptation of plants to drying soils. ABA caused a rapid depolarization of guard cell which is thought to result from anion efflux following activation of S-type (for slow-activating) anion

channels (Mäser et al., 2003). In the plasma membrane of guard cells, K_{in} channels are inhibited by ABA and K_{out} channels are activated by ABA (Pandey et al., 2007). ABA-induced membrane depolarization coupled with the up-regulation of K_{out} channel activity induces net K^+ efflux from guard cells; the consequence of which is loss of cell turgor and stomatal pore closure (Hosy et al., 2003). However, in roots, water stress and ABA modified the permeability of plasma membrane and significantly down-regulated the activity of K_{out} channels and activated K_{in} activity in the root stelar cells but had no effect on K^+ channel activity in the root cortex (Roberts and Snowman, 2000). The regulation of K^+ channels in roots is opposite to that in guard cells suggesting that alternative mechanisms underlie the ABA regulation of K^+ channels in roots. So far most of the studies focused on regulation of ion channels by ABA in guard cells. Little is known about ABA regulation of ion channel activity in roots.

The aim of the study was to fill in the above gaps in our knowledge. We have selected seven barley genotypes contrasting in drought tolerance (based on assessment of agronomical and physiological traits in response to water deficit reported in previous chapters) to elucidate the ionic (K^+, Na^+ and Cl^-) mechanisms of plant adaptive responses to hyperosmotic stress. We also reveal the role of abscisic acid in the regulation of ion fluxes in barley roots.

6.2 Results

6.2.1 Profiling steady net K^+, Na^+ and Cl^- fluxes along the root

It was expected that functionally different root zones would show different ion flux patterns under hyperosmotic stress. To test this hypothesis, we measured steady state K^+, Na^+ and Cl^- fluxes from the root epidermis of ZUG293 (drought tolerant) genotype. The measurements were taken from 0.5mm to 50mm distance from the root tip covering all roots zones. Under control, there was a net efflux of K^+ between 0.5mm and 4mm distance from the root tip while the root between 5mm and 50mm had a net uptake of K^+. The highest efflux (-444 nmol $m^{-2}s^{-1}$) was measured at 2mm and the highest influx (135 nmol $m^{-2}s^{-1}$) was measured at 20mm. Hyperosmotic stress (200mM mannitol) caused significant variation of K^+ in all root zones as compared to control. There was a continuous potassium efflux between 0.5mm (-350 nmol $m^{-2}s^{-1}$) and 4mm (-56 nmol $m^{-2}s^{-1}$) from the root tip. However, net K^+ remained positive (inward directed) between 5mm and 50mm. In the mature root zone, the influx of potassium was highest at 20mm followed by 30mm (285 nmol $m^{-2}s^{-1}$ and 197 nmol $m^{-2}s^{-1}$ respectively) (Fig 6.1A).

In case of Na^+ flux measurements, all the root zones showed Na^+ efflux under control conditions ranged between -35 nmol $m^{-2}s^{-1}$ (10mm) to -84 nmol $m^{-2}s^{-1}$ (20mm). Hyperosmotic stress induced significantly bigger Na^+ extrusion (Fig 6.1B). Net Na^+ efflux was highest at 15mm (-136 nmol $m^{-2}s^{-1}$) followed by -113 nmol $m^{-2}s^{-1}$ at 20mm. The least efflux of Na^+ was measured at 3mm (-67 nmol $m^{-2}s^{-1}$) (Fig 6.1B).

Under control, there was a net efflux of Cl in between 0.5mm and 2mm. The highest efflux was recorded at 1mm (-1428 nmol $m^{-2}s^{-1}$) while between 3mm and 50mm, a continuous Cl uptake was measured with the highest influx at 4mm (854 nmol $m^{-2}s^{-1}$) (Fig 6.1C). Hyperosmotic stress caused a decrease in efflux as compared to control between 0.5mm and 2mm root and overall increase in uptake at 3mm and between 10mm and 50mm. The highest increase in Cl^- uptake was at 20mm (889 nmol $m^{-2}s^{-1}$) followed by 30mm (845 nmol $m^{-2}s^{-1}$) from the root tip.

Based on these measurements, two most responsive root sites were selected for further studies (4mm in elongation zone and 20mm in mature zone).

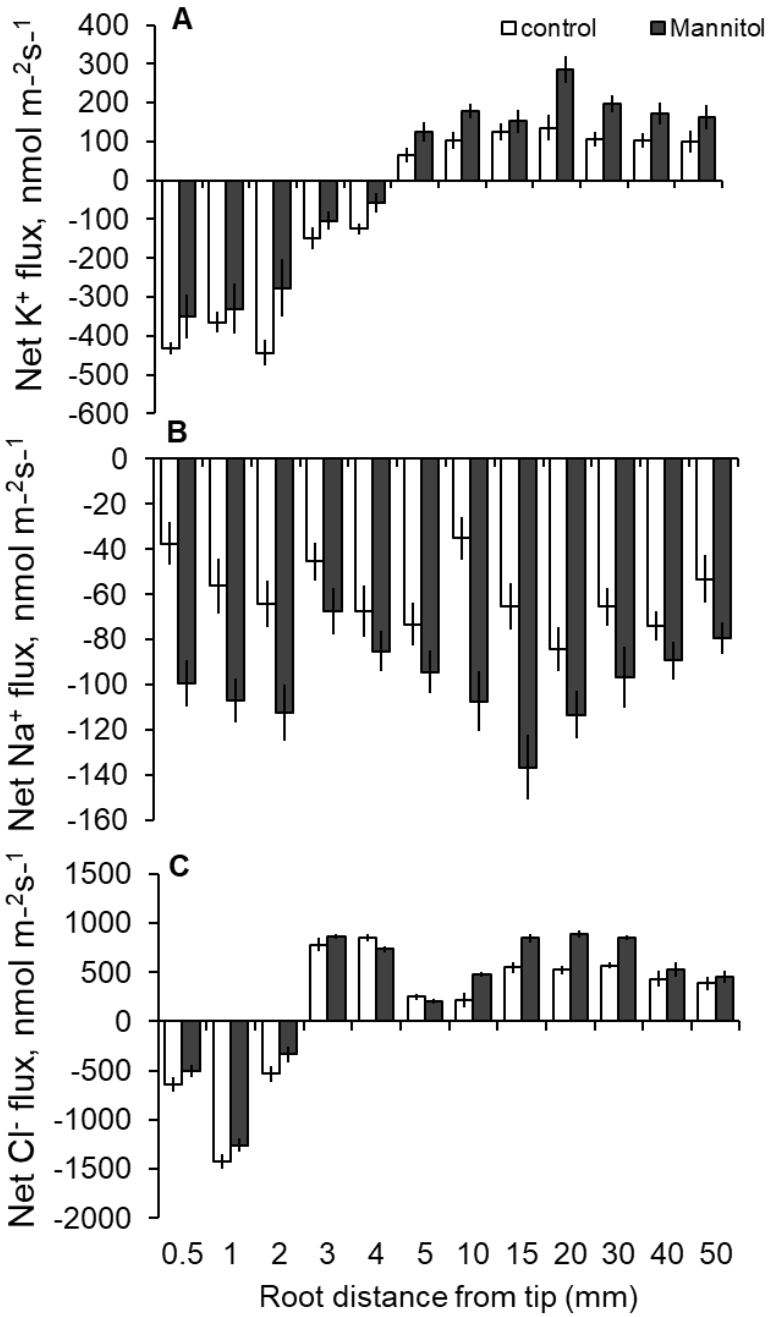

Figure 6.1 Net steady-state K^+ (A), Na^+ (B), Cl^- (C) measured from the epidermal root surface at various positions between 0.5mm to 50mm from the tip of ZUG293 barley genotype grown under control (BSM) and 200mM mannitol for 24 hours. Data is mean ±SE (n=6)

6.2.2 Dose-dependency of ion flux responses to osmotic stress

Before comparing the genotypic differences in root ion fluxes responses between contrasting barley genotypes to hyperosmotic stress, we have decided to select the suitable mannitol concentration at which plant roots could have maximum uptake of ions. Accordingly, dose dependence experiments were performed in two functionally different root zones (elongation and mature) of two drought contrasting genotypes (ZUG293-tolerant and Franklin-sensitive) in response to increasing concentration of mannitol. The results revealed that ZUG293 was losing K^+ under control and all mannitol treatments in the elongation zone. However, Franklin was losing K^+ under control, 20mM and 50mM concentrations of mannitol. There was an uptake of K^+ by the application of 100mM to 400mM mannitol concentrations in the root elongation zone of Franklin (Fig 6.2A). In mature zone, both genotypes showed K^+ influx, however, ZUG293 have taken more K^+ (154 nmol $m^{-2}s^{-1}$) compared to Franklin (40 nmol $m^{-2}s^{-1}$) in response to 200mM mannitol (Fig 6.2D). Under control conditions, both genotypes showed Na^+ influx in the elongation and mature root zones. Hyperosmotic stress also induced Na^+ efflux for both genotypes in elongation and mature zone (Fig 6.2B&E). Franklin and ZUG293 lost maximum Na in response to 200Mm (-58 nmol $m^{-2}s^{-1}$and -76 nmol $m^{-2}s^{-1}$respectively) in elongation zone. However, in mature root zone ZUG293 and Franklin lost highest Na^+ in response to 400mM mannitol (-30 nmol $m^{-2}s^{-1}$). In elongation zone, ZUG293 and Franklin had an uptake of Cl^- in response to 200mM and 400mM mannitol (Fig 6.2C). In the mature zone, mannitol treatments from 50mM to 400mM caused net chloride influx for both genotypes (Fig 6.2F). 200mM mannitol in mature zone being the most responsive concentration was selected for further experiments.

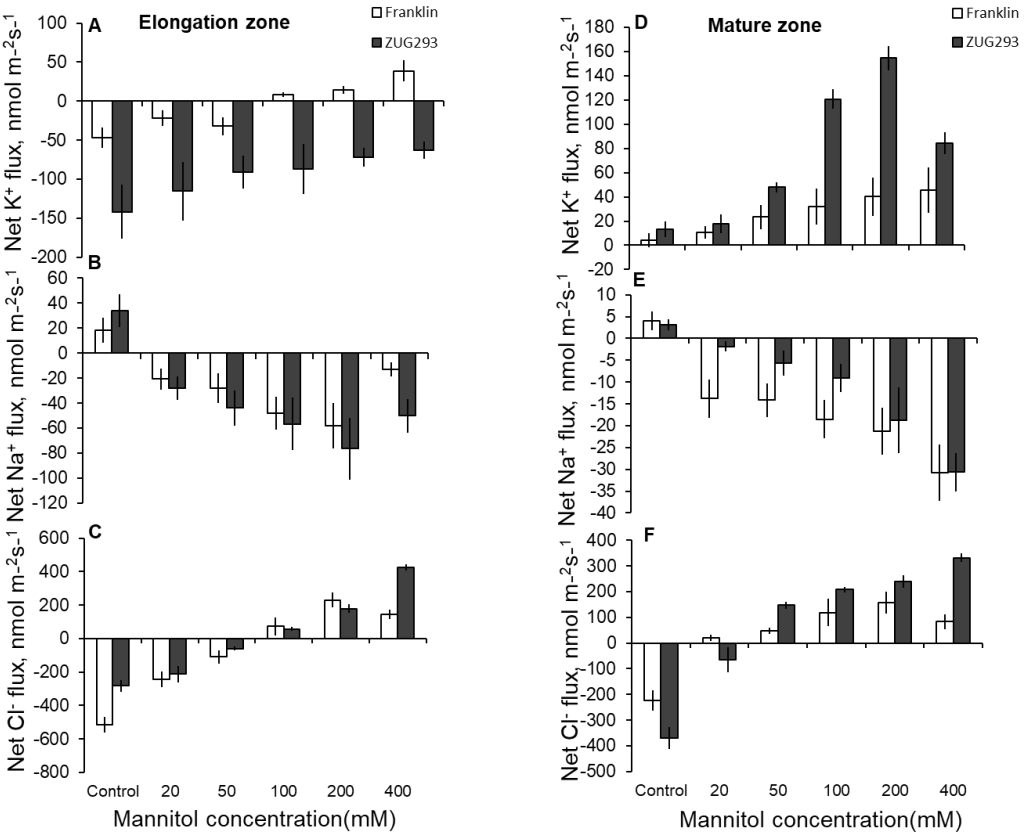

Figure 6.2 Net K^+, Na^+ and Cl^- fluxes under control and in response to 20, 50, 100, 200, and 400mM mannitol. Net fluxes were measured in root elongation (A, B, C) and mature zone (D, E, F) of Franklin and ZUG293. Data is mean ±SE (n=6)

6.2.3 Comparing transient ion responses of seven contrasting genotypes

Hyperosmotic stress (200mM mannitol) caused immediate changes in transient ion fluxes of seven barley genotypes by shifting K^+ and Cl^- fluxes from net efflux to net uptake. Transient K^+, Na^+ and Cl^- ion fluxes of two most representative genotypes, ZUG293 and Gairdner are shown in Fig 6.3A, B & C. The net influx of K^+ for all genotypes varied between 103 nmol $m^{-2}s^{-1}$ (Numar) and 10 nmol $m^{-2}s^{-1}$ (Gairdner) (Fig 6.4A). Na^+ showed differential responses under hyperosmotic stress, with larger efflux in X123 up to -102 nmol $m^{-2}s^{-1}$ and smallest up to -15 nmol $m^{-2}s^{-1}$ in Fleet (Fig 6.4B). Hyperosmotic stress caused a significant shift towards the net Cl^- influx with highest uptake found in ZUG293 (242 nmol $m^{-2}s^{-1}$) and lowest by Gairdner (61 nmol $m^{-2}s^{-1}$) (Fig 6.4C). Net ion fluxes in response to 200mM mannitol in all barley genotypes were correlated with the drought damage index. The correlational analysis showed a strong negative correlation between net K^+ fluxes under hyperosmotic stress condition and drought damage index (R^2=0.79; significant at $P<0.05$) (Fig 6.4D). However, no correlation was found between net Na^+ fluxes for all genotypes under stress and drought damage index (Fig 6.4E). A strong negative correlation was seen between damage index and net Cl fluxes in response to hyperosmotic stress (R^2=0.77*;* significant at $P<0.05$) (Fig 6.4F).

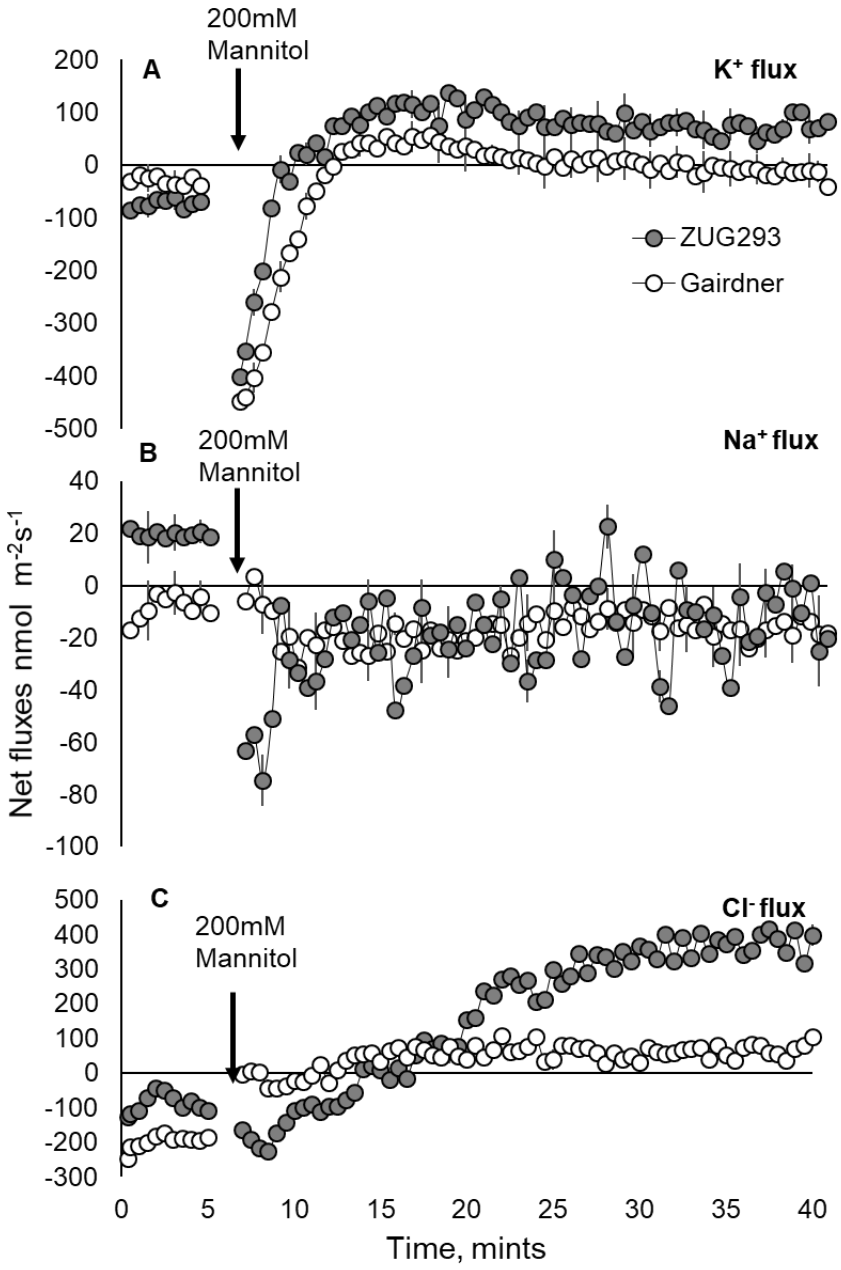

Figure 6.3 Transient K^+ (A), Na^+ (B) and Cl^- (C) fluxes from two contrasting barley genotypes ZUG293 and Gairdner in response to hyperosmotic stress (200mM mannitol added at 5th minute). Data is mean ±SE (n=6). Each point represents the running average of six means averaged during 30s intervals.

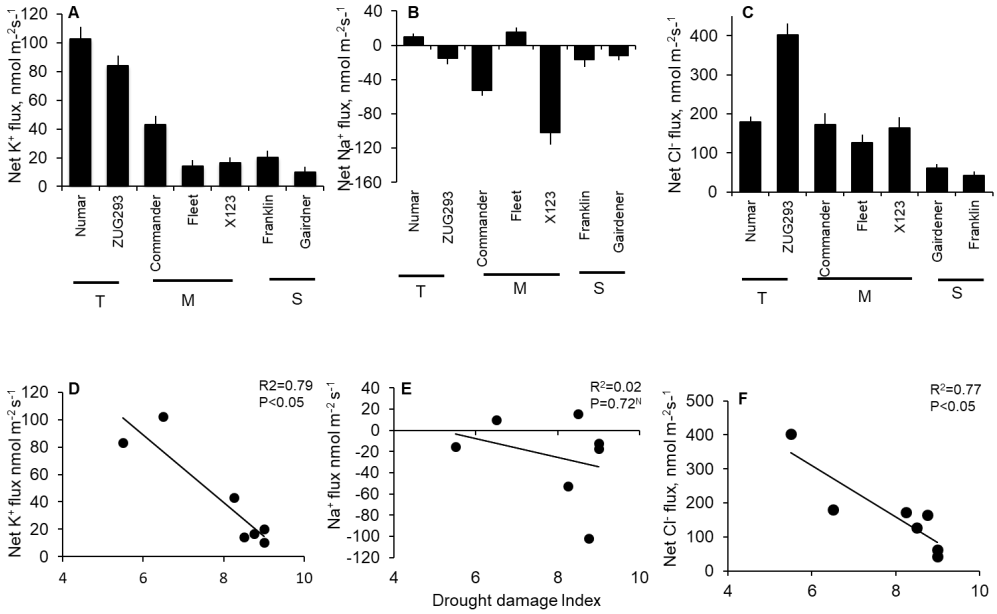

Figure 6.4 Net K^+ (A), Na^+ (B) and Cl^- (C) fluxes measured from mature root zone of seven barley genotypes contrasting in drought tolerance in response to 200mM mannitol. Data is mean ±SE (n=6). Correlation between drought damage index and net K^+ (D), Na^+ (E) and Cl^- (F) steady fluxes

6.2.4 Long-term hyperosmotic stress caused further increase in uptake of K^+ and Cl^-

The huge difference in transient K^+ and Cl^- influx among the drought tolerant and sensitive genotypes has led to further investigation of ions homeostasis upon long term hyperosmotic exposure (48h of mannitol treatment). The results showed that long term hyperosmotic stress showed more pronounced increased in the uptake of K and Cl (Fig 6.5A & C) in tolerant genotypes relative to net uptake measured after immediate application of mannitol (Fig 6.4). Drought tolerant ZUG293 had taken highest K^+ (310 nmol $m^{-2}s^{-1}$) while X123 had taken least K^+ (49 nmol $m^{-2}s^{-1}$) (Fig 6.5A). Fleet, Gairdner and Commander showed a small Na^+ uptake (36, 16 and 14 nmol $m^{-2}s^{-1}$ respectively) but all other genotypes including drought tolerant ZUG293 and Numar had lost Na^+ (Fig 6.5B). However, ZUG293, Numar, Commander and X030 had high influx of Cl^-, the highest Cl^- uptake was found in ZUG293 (533 nmol $m^{-2}s^{-1}$) whereas Franklin, Fleet, Gairdner and X123 lost Cl^-. Franklin lost the maximum Cl^- (-600 nmol $m^{-2}s^{-1}$) (Fig 6.5C). A strong negative correlation was observed between the drought damage index and K^+ and Cl- fluxes under hyperosmotic stress for 48hours (R^2=0.60; R^2=0.66 significant at *P<0.05*) (Fig 6.5D). There was a strong positive correlation

between drought damage index and net Na^+ flux (R^2=0.57*;* significant at *P<0.05*) (Fig 6.5E).

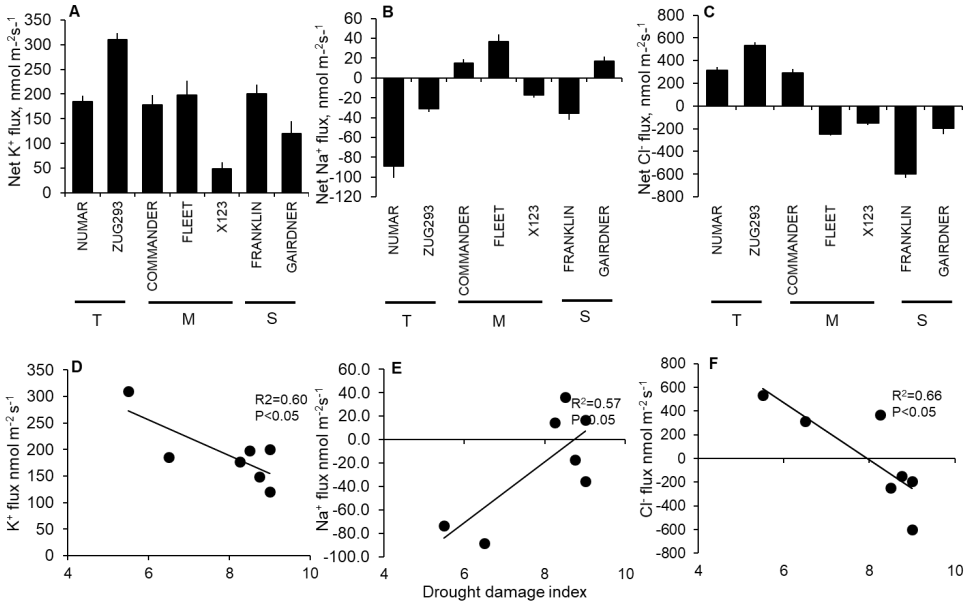

Figure 6.5 Steady-state net K^+(A), Na^+(B) and Cl^-(C) fluxes of seven barley genotypes contrasting in drought tolerance after 48 hours of hyperosmotic stress (200mM mannitol). Data is mean ±SE (n=6). Correlation between drought damage index and net K^+ (D), Na^+ (E)and Cl^-(F) steady fluxes.

6.2.5 Drought-tolerant genotypes maintain a more negative membrane potential under hyperosmotic stress

As many K^+ transporters are known to be voltage gated (Ward et al. 2009), the difference in K^+ uptake reported above could be attributed to the difference in ability to control cell membrane potential (MP). This issue was studied by measuring MP values of root epidermal cells of intact plants from mature root zone treated with 200mM mannitol for 48 hours. The membrane potential of all genotypes varied between -111mV and -130mV in control. Hyperosmotic stress caused significant hyperpolarization of plasma membrane except X123 as compared to control (as illustrated in Fig 6.6A). The highest membrane potential values under hyperosmotic stress was measured in drought tolerant genotype ZUG293 (-167mV) and the lowest was found in Gairdner (drought sensitive) (-94mV). A very strong (R^2=0.61*;* significant at *P<0.05*) correlation was found between the drought damage index and membrane potential values measured under hyperosmotic stress conditions (Fig 6.6B).

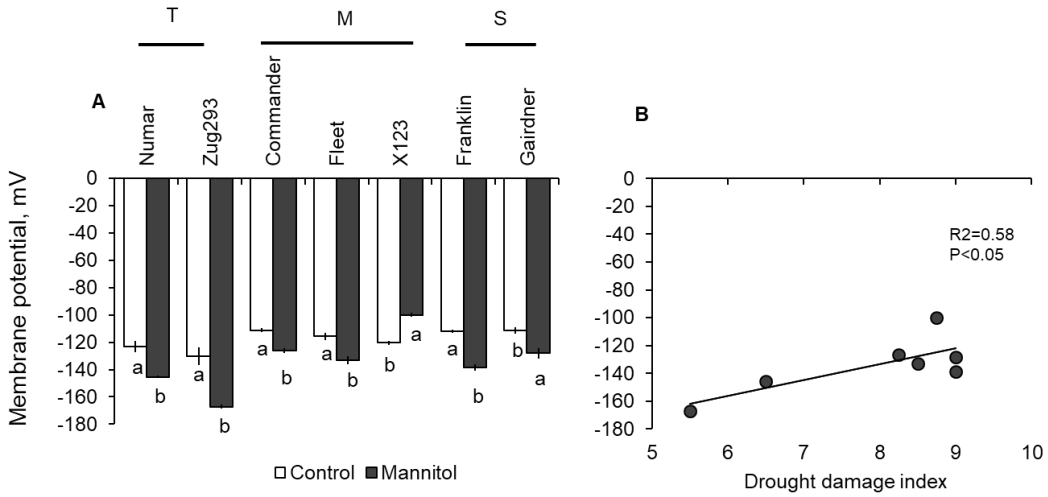

Figure 6.6 Effect of hyperosmotic stress (200mM mannitol) after 48 hours on the membrane potential (MP) of seven barley contrasting genotypes measured from mature zone (~20mm from the root tip) (A). Data are the mean ± SE (n=18) measurements from five individual plants (T-tolerant, M-moderate tolerant, S-sensitive). Different lower-case letters indicate a significant difference at *P≤0.05* according to Duncan's multiple range tests. Correlation between MP values and drought damage index (B)

6.2.6 ABA induced changes in ion-fluxes

Abscisic acid induces solute accumulation in the root under drought stress condition. By regulating different ion channels ABA facilitates the entry of cations especially K^+ by mediating K inward channels. To clarify the specific role of ABA in regulating ion channels, ion fluxes were measured in mature zone of root epidermis in response to acute 10µM ABA treatment. Surprisingly, 10µM ABA resulted in no significant change in K^+ and Na^+ fluxes in either of two barley genotypes compared with the ion fluxes measured without onset of ABA treatment (Fig 6.7D & E). However, Cl^- fluxes for both genotypes were strongly responsive to ABA treatment (Fig 6.7C). Before the onset of ABA, there was continuous efflux of Cl^- for both genotypes (-38 nmol $m^{-2}s^{-1}$ and -190 nmol $m^{-2}s^{-1}$ in ZUG293 and Gairdner respectively). However, the application of 10µM ABA resulted in sharp increase in net Cl^- influx into root epidermis in both ZUG293 and Gairdner; the net uptake was significantly *(P<0.05)* higher in a drought tolerant ZUG293 cultivar (195 and 79 nmol $m^{-2}s^{-1}$ in ZUG293 and Gairdner respectively) (Fig 6.7F).

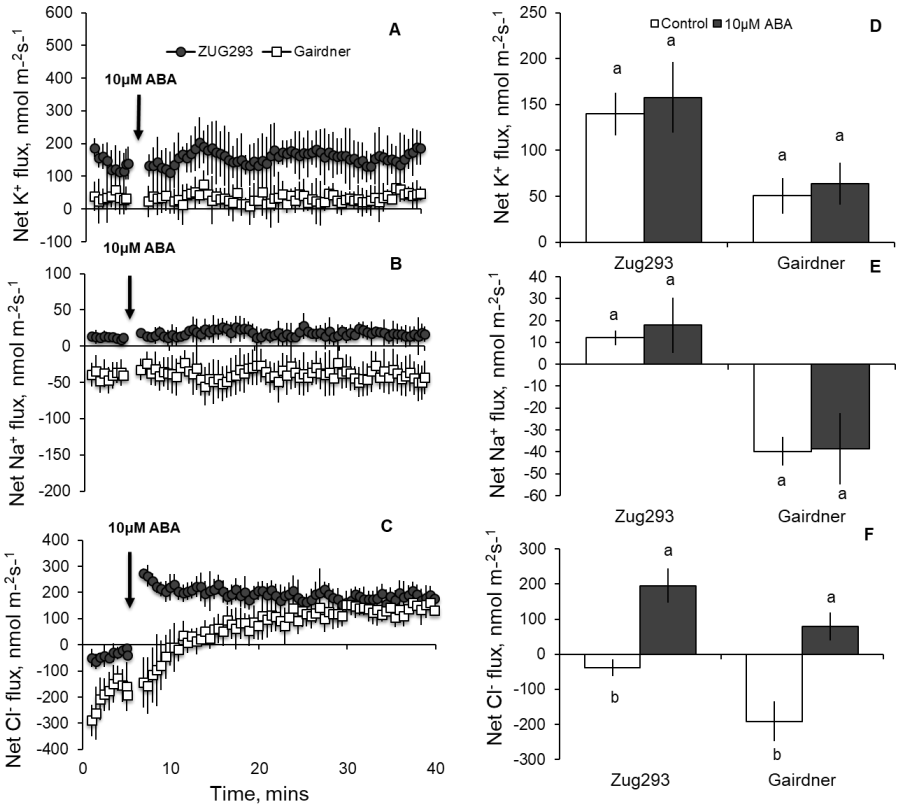

Figure 6.7 Transient K^+ (A), Na^+ (B) and Cl^-(C) flux responses measured from root mature zone in ZUG293 and Gairdner in response to 10µM Abscisic acid (ABA). Mean ±SE (n=6). Net K^+ (D), Na^+ (E) and Cl^-(F) fluxes after 10µM ABA treatment. Different lowercase letters indicate significant difference at $P \leq 0.05$ between control and ABA treatment in two barley genotypes i.e., ZUG293 (tolerant) and Gairdner (sensitive)

6.3 Discussion

6.3.1 Hyperosmotic stress induced uptake of K^+ and Cl^-

Under osmotic stress plants readjust their osmotic potential either by enhanced uptake of inorganic ions or by de novo synthesis of compatible solutes. Inorganic ions are used for cell osmotic adjustment due to their immediate cell turgor recovery and also because of their low energetic cost (Shabala and Shabala, 2011). Therefore, mostly plants rely on inorganic ions as a metabolically cheap osmoticum for osmotic adjustment. Previously Lew (1998) found that 200 mM mannitol caused an obvious K^+ loss in Arabidopsis root hairs with no significant changes in Cl^- flux. On the contrary, mannitol caused a dramatic K^+ and Cl^- uptake in bean mesophyll cells and barley roots (Chen et al., 2005; Shabala et al., 2000; Shabala and Lew, 2002).We showed that mannitol treatment caused an immediate uptake of K and Cl, with much more uptake of K in drought tolerant genotypes (ZUG293, Numar) as compared to drought sensitive genotypes (Franklin, Gairdner) (Fig 6.4). Long term osmotic stress induced further increase in K in all genotypes while tolerant genotypes showed increase in uptake of Cl and moderately tolerant and sensitive genotypes had lost Cl. This genotypic difference in K uptake under osmotic stress is also reported in another study where tolerant barley genotypes were able to maintain K uptake under short and long-term drought and hyperosmotic stress as compared to drought sensitive genotypes of barley (Feng et al., 2016). A plausible hypothesis to explain the dramatic uptake of K in barley roots upon osmotic treatment could be the activation of HvAKT1 (KIR channels) as a result of MP hyperpolarization brought about by increased uptake of Cl or restriction of KOR (outward-rectifying K^+ channels) at the plasma membrane (Gierth and Mäser, 2007; Shabala et al., 2000). AKT1 overexpression improved osmotic and drought stress tolerance by increasing levels of K^+ in root tissues (Ahmad et al., 2016). Another possibility for the uptake of K^+ is via HvHAK1 (HAK/KUP/KT transporters) which cotransport K^+ and H^+ (Santa-María et al., 1997). H^+/K^+ and H^+/Cl^- symporters which are known to be present at the plasma membrane (Felle, 1994; Maathuis and Amtmann, 1999) and activate by the extrusion of H^+ ions when the plasma membrane hyperpolarized under hyperosmotic (see below in detail)

6.3.2 Membrane potential is hyperpolarized under hyperosmotic stress and MP values are positively correlated with drought tolerance

The plasma membrane is responsible for the maintenance of ionic and electric gradients between the cytosol and external media and therefore crucial for intracellular ionic homeostasis (Gill, Muhammad B et al., 2017). It is also an important component of the signal transduction in plants under stress conditions (Tuteja and Sopory, 2008b). Channel mediated transport of ions depends on the electrical potential difference across the PM controlled by H^+-ATPase activity (Palmgren and Nissen, 2011). H^+ pumps also create proton motive force for driving secondary active transport of ions (Haruta et al., 2015). We showed that hyperosmotic stress caused rapid and significant hyperpolarization of plasma MP (-156mV to -133mV range) in all barley genotypes compared to control (-117mV) (Shabala and Lew, 2002). Under hyperosmotic stress, the plasma membrane H^+-ATPase activity is regulated by subsequent binding of 14-3-3 protein (phosphopeptide-binding proteins) to the autoinhibitory C-terminal domain of the pump and such binding requires phosphorylation of the penultimate threonine residue (T947 in AHA2) (Cotelle and Leonhardt, 2016; Fuglsang et al., 1999). H^+-ATPase-mediated H^+ efflux from the cytosol hyperpolarizes the membrane potential beyond the equilibrium potential for K^+ and activates HvAKT1/HvHAK1, leading to K^+ influx (Pandey et al., 2007) or alternatively outward K^+ channels may be partially shut down and reducing K efflux (Lew, 1996). It was reported earlier that in barley (recombinant 14-3-3B) and overexpression of a tomato 14-3-3 homologue (GRF9) resulted in an increased H^+-ATPase activity during water stress (He et al., 2015; Van den Wijngaard et al., 2005). Steeper H^+ gradients created by H^+ ATPase also lead to increased uptake of anion Cl^- as a result of increased driving force of proton coupled symport systems (H^+/Cl^-) (Shabala and Lew, 2002).We also found that drought tolerant genotypes maintained more negative membrane (-145 to -168 mV range) potential under hyperosmotic stress as compared to drought sensitive genotypes (-128 to -138 mV range). It is evident that drought tolerance or tolerance to osmotic stress is highly associated with membrane hyperpolarization as a result of increased H^+-ATPase activity as shown by earlier studies on wheat and oat that early and increased activation of PM H^+-ATPase activity were found in drought tolerant genotypes compared to drought sensitive genotypes (Gong et al., 2010; Liu et al., 2005). This

increased PM H^+-ATPase activity triggers the increased biosynthesis of major osmolytes, which, in turn, leads to the up-regulation of water maintenance system.

6.3.3 Mature zone had increase uptake of ions

Epidermal cells from elongation and mature root zones were previously shown to have distinctly different ion transport patterns reflecting differences in either the functional expression or gating properties of major ion transporters (Foster and Miklavcic, 2016; Zhou et al., 2011). Our results indicated that mature root zone had more consistent uptake of K and Cl (Fig 6.2) compared to root apex and elongation zone (Chen et al., 2005; Itoh et al., 1986). The increase in uptake of nutrients relatively in mature zone could be due to more negative membrane potential towards mature zone and increased H^+ activity in the mature zone. In a comparison study between root apex and mature root zone, it was revealed that even under control conditions, the membrane potential values of mature cells were more negative compared to apex cells and mature zone showed higher potency for repolarization (Shabala et al., 2016). Moreover, the same study showed that the mature zone exhibited a higher H^+-pumping capacity compared with apex cells. We can hypothesize that under hyperosmotic stress the root mature zone might be more hyperpolarized as we conducted MP measurements only in mature zone of the root. More work needs to be done in the future to reveal the effect of hyperosmotic stress in all root zones.

6.3.4 ABA had no significant effect on cations but regulated the Cl uptake

Under drought stress, ABA synthesis and signalling lead to stomatal closure via activation of anion channel concomitant with regulation of K^+ fluxes (Geiger et al., 2011; Kim et al., 2010; Mori and Murata, 2011). For instance, ABA decreases K^+ influx by deactivating inward channels and increases K^+ efflux through activating outward K^+ channels in guard cells (Leyman et al., 1999; Schwartz et al., 1994). One of the inward-rectifier K^+ channel subunits, AKT1, has been demonstrated to play an important role in K^+ uptake at the root (Alemán et al., 2011; Rubio et al., 2008) and also has been well expressed in stomata (Szyroki et al., 2001). More importantly, AKT1 is activated by phosphorylation through the CIPK23–CBL1/9 complex (Xu et al., 2006). Previous studies have revealed that the absence of an active AKT1 due to a mutation in the gene encoding the channel itself (*akt1* line) or in a kinase that enhances

its activity (*cipk23* line) leads to an improved stomatal closure in response to ABA relative to wild plants (Nieves-Cordones et al., 2011) suggesting that K^+ inward-rectifying currents, like those mediated by AKT1, down-regulated after ABA exposure in guard cells, promoting stomatal closure.

Contrary to guard cells, ABA application increased the uptake of chloride ions in root mature zone of both drought tolerant and sensitive genotypes (Fig 6.7C & F). The results were unexpected given the reported evidence that ABA promotes Cl^- efflux via SLAH3 in root epidermal cells (Planes et al., 2014; Roelfsema et al., 2012). The possible justification could be the increased activity of H^+ ATPase in roots induced by ABA which could hyperpolarized the plasma membrane (Planes et al., 2014). Using the chemical energy of ATP, the plasma membrane H^+-ATPases extrude protons from cells to generate an electrochemical proton gradient. The activation of H^+ pump and resulting extrusion of H^+ ions may enhance chloride uptake via cotransport mechanism of H^+/Cl^- symporter present at plasma membrane (Felle, 1994; Shabala and Lew, 2002). Previous studies suggested that moderate water stress increased root-tip accumulation of ABA and the ABA signaling modulates the auxin transport which activates the PM H^+ ATPase to release more protons in the root tip (Xu et al., 2013). Similarly, in another study it was seen that 24-hour treatment of cucumber roots with ABA enhanced the activity of PM-H^+ ATPase but interestingly when ABA was added to the reaction medium there were no observed changes in ATPase activity which indicated that plasma membrane activity of proton pumping could be at the level of gene expression (Janicka-Russak and Kłobus, 2007).

However, ABA had no significant effect on cations (K^+ and Na^+) in barley roots. It was reported in a study on maize that ABA regulation of K^+ channel activity in maize root stele was opposite to that observed in guard cells and showed no effect on K^+ channel in root cortex suggesting that ABA does not regulate low affinity K uptake in root cortex (Roberts, 1998). In addition to this, we have measured net fluxes of ions in MIFE technique therefore there could be a possibility that effect of ABA on low affinity channels (AKT1) were compensated by the effect of ABA on HAK/KUP/KT transporters and hence the net flux we measured was not significantly different from the flux measured under control conditions.

Chapter 7. General discussion

Drought stress is one of the major abiotic stresses restricting plant growth and productivity worldwide. To ensure global food security under current climate trends, a major breakthrough in crop breeding for drought tolerance is required. The complex nature of plant drought tolerance, the shortage of reliable and comprehensive screening methods, and the lack of a comprehensive understanding of the underlying physiological mechanisms of drought tolerance hinder a further improvement in selecting and breeding for drought-tolerant crop species. Barley and wheat are two major crops cultivated world-wide; both of them experience large yield penalties from drought in their production habitats. Due to significant genetic variabilities, barley and wheat exhibit a plethora of morphological and physiological responses to drought stress and rely on different adaptive mechanisms. These adaptive mechanisms involve root traits, stomatal regulations and osmotic adjustment (Ashraf et al., 2011; Farooq et al., 2009b; Hussain Wani et al., 2013). Highlighting the above, the present research into whole-plant and physiological response to drought has revealed several aspects of drought tolerance.

In order to advance our knowledge on the adaptive mechanisms underlying drought tolerance, we evaluated different adaptive mechanisms at whole plant level using seven barley genotypes (selected from screening experiments) contrasting in their ability to tolerate drought stress. These genotypes were exposed to control and two water deficit regimes (25% and 12% of full field capacity). In general, root length, relative water content, stomatal conductance, root K and Cl, leaf Cl, total soluble sugars and total amino acids were the main factors contributing drought tolerance under severe drought stress. This indicates that tolerant genotypes developed longer roots as they absorb water from deeper soil layers (Hu and Schmidhalter, 2005; Wang et al., 2013). Also, tolerant genotypes had high stomatal conductance rates to maximize carbon assimilation for improved photosynthetic rate (Broadley, 2012; Egilla et al., 2001). The tolerant genotypes with high Gs were able to maintain high relative water content which can be attributed to longer roots and better osmotic adjustment in these genotypes compared to moderately tolerant and sensitive genotypes of barley. Plants generally undergo osmotic adjustment in roots and/ or leaves either by increased uptake of inorganic ions or by accumulation of comptible solutes. Our results showed

that tolerant genotypes had relatively more K in root and high Cl in root and leaf under drought stress compared to moderately tolerant and sensitive genotypes. Though total soluble sugars and total amino acids were also high in tolerant genotypes, however, the percent contribution towards the osmotic adjustment was more by inorganic ions in the order Cl > K > TSS > TAA > Na. As the energy cost for uptake and compartmentation of inorganic ions is much lower as compared to *de novo* synthesis of compatible solutes (Raven, 1985), stronger reliance on inorganic osmolytes for osmotic adjustment has made more of energy (ATP stored) available for growth, explaining better performance of the tolerant genotypes.

Another important finding was that stomatal conductance was closely related to leaf and root Cl (Fig 5.14) which reflect an intrinsic cascade of Cl^- transport from root to shoot in drought stress. Under drought stress when energy is limiting factor for plants, Cl acts as a beneficial nutrient due to that limits the energy costs associated with malate biosynthesis and stomatal opening (Van Kirk and Raschke, 1978). The improved stomatal regulation by Cl and K facilitates high rate of photosynthesis which consequently increase the plant biomass under drought conditions (Broadley, 2012; Egilla et al., 2001). The observed increase in leaf Cl in our experiments is associated with increased Cl uptake in roots. Cl^- is ubiquitous in nature and actively taken by higher plants (Broadley, 2012; Franco-Navarro et al., 2015) compared to K uptake which comes by passive way. Under drought stress, root plasma membrane depolarisation limits passive K uptake (Shabala et al., 2005) but at the same time decrease the steepness of electric gradient preventing Cl^- entry (Broadley, 2012). Hence, plants could rely more heavily on Cl than K for cell osmoregulation (including in guard cells) as shown by our results in which chloride represented the highest contribution in osmotic adjustment followed by K in tolerant genotypes.

While studying ionic mechanisms in response to hyperosmotic stress using the non-invasive ion-selective microelectrode measurements (the MIFE technique), we observed that tolerant genotypes had high K^+ and Cl^- in response to hyperosmotic stress compared to moderately tolerant and sensitive genotypes. This phenomenon was attributed to activation of HvAKT1 uptake channels and HvHAK1 (HAK/KUP/KT transporters) in tolerant genotypes, as evident from the fact that they were able to maintain more negative membrane potential under hyperosmotic stress (Chapter 6). Both HvAKT1/HvHAK1 activated by membrane hyperpolarization brought about by increased uptake of Cl^- (Gierth and Mäser, 2007; Santa-María et al., 1997; Shabala et

al., 2000). Channel mediated transport of ions depends on the electrical potential difference across the PM controlled by H^+-ATPase activity (Palmgren and Nissen, 2011). H^+ pumps also create proton motive force for driving secondary active transport of ions (Haruta et al., 2015). Another important finding is that effects of abscisic acid (ABA) on K^+ transport in root cells was different from that in guard cells. Application of ABA induced an increase in Cl^- in both tolerant and sensitive genotypes and caused no significant effect on cations (K^+ and Na^+). The increase in chloride uptake is generally promoted by increased activity of H^+ ATPase in roots induced by ABA which could hyperpolarized the plasma membrane (Planes et al., 2014). Using the chemical energy of ATP, the plasma membrane H^+-ATPases extrude protons from cells to generate an electrochemical proton gradient. The activation of H^+ pump and resulting extrusion of H^+ ions may enhance chloride uptake via cotransport mechanism of H^+/Cl^- symporter present at plasma membrane (Felle, 1994; Shabala and Lew, 2002).

Lack of convenient and reliable screening techniques significantly handicapped the progress in plant breeding. Different screening techniques were assessed in this research. Plants were visually evaluated by assigning drought damage index (0=damage, 10= all plant dead) based on counting total number of leaves and total number of chlorotic and necrotic leaves at three-time points. This visual evaluation is a traditional method to measure drought tolerance and did not require any equipmental expertise. Moreover, this method is a simple, cheaper and feasible technique to measure drought tolerance of a large germplasm. Also, we can recommend breeders to conduct screening by using relatively large tanks under which plants are more gradually exposed to water deficits compare to small pots, thus providing more uniform background and increasing reliability of the procedure. The suitability of various physiological (chlorophyll content, stomatal conductance, chlorophyll fluorescence, relative water content) traits and biomass to be used as drought indices were evaluated under three different water regimes (control, 25% and 12% of full field capacity). Plant dry biomass provide reliable tolerance information as most of the drought tolerant barley and wheat genotypes with lowest drought damage index accumulated relatively more dry weight under drought stress conditions and the genotypes with highest damage index accumulated lowest shoot dry weight. The measurements of chlorophyll content (SPAD values) and maximum quantum efficiency of light harvesting in PSII in dark adapted leaves (*Fv/Fm* ratio) were rapid

and non-invasive and therefore are suggested as most efficient and reliable physiological parameters for screening large number of genotypes in a very short time.

Following different screening techniques, we identified some highly drought tolerant and sensitive genotypes in barley and wheat germplasm (see in the last section) suggesting that the germplasm used in this study could be a rich source of genetic diversity for breeding purposes. It is known that barley is relatively tolerant crop to drought in context to yield potential compared to wheat. This study explored that both the crops indicated a similar range in drought tolerance and sensitivity by evaluating SPAD values as SPAD was found to be good indicator of drought tolerance in Chapter 3 & 4. However, the data of stomatal conductance and relative water content intimated that both barley and wheat had contrasting tolerance mechanisms for adapting drought stress at whole plant level. The barley genotypes showed high values of relative Gs and RWC compared to control. In wheat, tolerance is achieved by early closing the stomata to maintain relative water content. However, barley plants with high Gs achieved tolerance most likely by osmotic adjustment to maintain RWC. Though in this study only vegetative stage was considered, in the long term the more efficient osmotic adjustment could sustain high photosynthetic rate because of favourable relative water status and high stomatal opening in barley could have the potential to enhance crop productivity.

Future research and recommendations

Among studied barley genotypes collected from different geographical origins, Numar, Flagship, ZUG293, and X026 were found to be the most drought tolerant, with relatively low drought damage index, higher biomass accumulation, chlorophyll content, relative water content, stomatal conductance values. Franklin and Gairdner were found to be most sensitive to drought stress. These genotypes could be used as the parent lines for DH population/Near Isogenic Lines to find out the QTLs/genes conferring drought tolerance mechanisms. Among wheat, Mahon Demias, Albidum24, Tainong292 were regarded as the most tolerant to drought stress; while Kord Cl Plus, Onohoiskaja4 and Zhengmai9023 were the most sensitive to drought stress. It is recommended that these contrasting genotypes are used in further physiological (e.g. mechanisms) and genetic (QTL mapping) studies to improve drought stress tolerance in wheat.

The extension of this work may focus on the following aspects:

- Revealing QTLs for the major functional traits contributing to drought tolerance and, specifically, for those mechanisms involved in the osmotic adjustment.

- While the importance of activation of HvAKT1 and HvHAK1 to facilitate K uptake under hyperosmotic stress is well described in this study, more attention need to be paid in exploring the increased uptake of Cl^- in drought tolerant genotypes compared to sensitive genotypes. The key question to answer is of whether tolerant genotypes have improved selectivity of the Cl^- permeable proteins, or whether increased transcript level of these proteins in the root plasma membrane (PM) also plays a key role?

- In this work, we found that ABA regulation of ion transport in roots differed from that in guard cells. Further studies should investigate of whether ABA mediated ion fluxes in roots of other cereals also possess this pattern.

- This ABA mediated Cl^- uptake in the roots and the hyperpolarization of root PM both are intrinsically linked with H^+-ATPase activity. In the future, more comprehensive studies on the function of plasma membrane H^+-ATPase in response to hyperosmotic stress and ABA needs to be done.

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